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NEUROPROTECTION IN CEREBRAL ISCHEMIA: AN EFFECT OF c-JUN N-TERMINAL KINASE INHIBITION ON THE INFLAMMATORY RESPONSE

Corinne Benakis

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département des Neurosciences Cliniques

NEUROPROTECTION IN CEREBRAL ISCHEMIA: AN EFFECT OF c-JUN N-TERMINAL KINASE INHIBITION ON THE INFLAMMATORY RESPONSE

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par

Corinne BENAKIS

Biochimiste diplômée de l'Université de Genève, Suisse

Jury

Prof. Peter Clarke, Président
Dr Lorenz Hirt, Directeur
Prof. Richard Frackowiak, Co-Directeur
Dr Annemieke Kavelaars, Expert
Dr Julien Puyal, Expert
Prof. Jean-Pierre Hornung, Expert

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Experts	Madame Dr Annemieke **Kavelaars**
	Monsieur Dr Julien **Puyal**
	Monsieur Prof. Jean-Pierre **Hornung**

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Madame Corinne Benakis

master en biochimie Université de Genève

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NEUROPROTECTION IN CEREBRAL ISCHEMIA: AN EFFECT OF C-JUN N-TERMINAL KINASE INHIBITION ON THE INFLAMMATORY RESPONSE

Lausanne, le 6 mai 2011

pour Le Doyen
de la Faculté de Biologie et de Médecine
Peter A.H. Clarke
Prof. Peter Clarke

" […] par hasard, direz-vous peut-être, mais souvenez-vous que dans les champs de l'observation le hasard ne favorise que les esprits préparés […] "

Louis Pasteur

Œuvres de Pasteur, Tome VII, Mélanges scientifiques et littéraires
Index analytique et syntétique de l'œuvre de Pasteur,
MASSON & C^{IE} EDITEURS. PARIS.

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RESUME

Suite à un accident vasculaire cérébral (AVC) ischémique, les cellules gliales du cerveau deviennent activées, de nombreuses cellules inflammatoires pénètrent dans le tissu lésé et sécrètent une grande variété de cytokines et chémokines. Aujourd'hui, il existe des interrogations sur les effets bénéfiques ou délétères de cette inflammation sur la taille de la lésion et le pronostic neurologique.

Ce projet vise à évaluer l'effet d'un peptide neuroprotecteur, D-JNKI1, inhibiteur de la voie pro-apoptotique de signalisation intracellulaire c-Jun N-terminal kinase (JNK), sur l'inflammation post-ischémique.

Nous montrons d'abord que la microglie est largement activée dans toute la région lésée 48 h après l'induction d'une ischémie chez la souris. Cependant, malgré l'inhibition de la mort neuronale par D-JNKI1 évaluée à 48 h, nous n'observons de modification ni de l'activation de la microglie, ni de son nombre. Ensuite, nous montrons que le cerveau peut être protégé même s'il y a une augmentation massive de la sécrétion de médiateurs inflammatoires dans la circulation systémique très tôt après induction d'un AVC ischémique. De plus, nous notons que la sécrétion de molécules inflammatoires dans le cerveau n'est pas différente entre les animaux traités par D-JNKI1 ou une solution saline, bien que nous ayons obtenu une neuroprotection significative chez les animaux traités.

En conclusion, nous montrons que l'inhibition de la voie de JNK par D-JNKI1 n'influence pas directement l'inflammation post-ischémique. Ceci suggère que l'inhibition de l'inflammation n'est pas forcément nécessaire pour obtenir en haut degré de neuroprotection du parenchyme lésé après ischémie cérébrale, et que les mécanismes inflammatoires déclenchés lors d'une ischémie cérébrale ne sont pas forcément délétères pour la récupération du tissu endommagé.

SUMMARY

After cerebral ischemia, glial cells become activated and numerous inflammatory cells infiltrate the site of the lesion, secreting a large variety of cytokines and chemokines. It is controversial whether this brain inflammation is detrimental or beneficial and how it influences lesion size and neurological outcome.

This project was aimed at critically evaluating whether the neuroprotective peptide D-JNKI, an inhibitor of the pro-apopotic c-Jun N-terminal kinase (JNK) pathway, modulates post-ischemic inflammation in animal models of stroke. Specifically, it was asked whether JNK inhibition prevents microglial activation and the release of inflammatory mediators.

In the first part of this study, we showed that microglia was activated throughout the lesion 48 h after experimental stroke. However, the activation and accumulation of microglia was not reduced by D-JNKI1, despite a significant reduction of the lesion size. In the second part of this project, we demonstrated that neuroprotection measured at 48 h occurs even though inflammatory mediators are released in the plasma very early after the onset of cerebral ischemia. Furthermore, we found that secretion of inflammatory mediators in the brain was not different in groups treated with D-JNKI1 or not, despite a significant reduction of the lesion size in the treated group.

Altogether, we show that inhibition of the JNK pathway using D-JNKI1 does not influence directly post-stroke inflammation. Inhibition of inflammation is therefore not necessarily required for neuroprotection after cerebral ischemia. Thus, post-stroke inflammation might not be detrimental for the tissue recovery.

LIST OF ABBREVATIONS

AMPA	Amino-Methyl-Propanoic Acid
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
ATP	Adenosine Tri-Phosphate
BBB	Blood Brain Barrier
Bcl-2	B-Cell Lymphoma-2
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CA1	*Cornus Ammonis*-1
CBF	Cerebral Blood Flow
CD11b	Cluster of Differentiation molecule-11b
CINC	Cytokine-Induced Neutrophil Chemoattractant
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
CTRL	Control
d	days
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole
DC	Dendritic Cells
DIV	Days *In Vitro*
D-JNKI1	D-JNK Inhibitor-1
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribo Nucleic Acid
DTT	Dithiothreitol
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Fetal Carf Serum
GFAP	Glial Fibrillary Acidic Protein
GROα	Growth-Regulated Oncogene-α
h	hours
HBSS	Hank's Buffered Salt Solution
HEPS	Hydroxyethyl-Piperazineethane Sulfonic acid
HIV	Human Immunodeficiency Virus
HPA	Hypothalamic-Pituitary-Adrenal
IB-1	Islet Brain-1
IB4	Isolectin B4
ICAM-1	Inter-cellular Adhesion Molecule-1
IL	Interleukin
IL6-ra	IL6 Receptor monoclonal Antibody
iNOS	inducible Nitric Oxyde Synthase
IRDye	Infrared-Dye
i.v.	intra-venously
JAK-STAT	Janus Kinase-Signal Transducer Activator of Transcription
JBD	JNK Binding Domain

JIP	JNK Interacting Protein
JNK	c-Jun NH_2-terminal Kinases
KC	Keratinocyte-derived Chemokine
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCA	Middle Cerebral Artery
MCAO	Middle Cerebral Artery Occlusion
MCP1	Monocyte Chemotactic Protein-1
MEM	Minimum Essential Medium
MERCI	Mechanical Embolus Removal in Cerebral Ischemia
MHC II	Major Histocompatibility Complex II
min	Minutes
MKK, MEK, MAP2K	MAP Kinase Kinase
MKKK, MEKK, MAP3K	MAP Kinase Kinase Kinase
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
NeuN	Neuronal Nuclei
NGS	Normal Goat Serum
NMDA	N-Methyl-D-Aspartic acid
NS	Not Significant
OHC	Organotypic Hippocampal slice Culture
OGD	Oxygen and Glucose Deprivation
PBR	Peripheral Benzodiazepine Receptor
PBS	Phosphate Buffer Solution
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PI	Propidium Iodide
RAS	Rat Sarcoma
rpm	revolutions per minute
RT	Room Temperature
rtPA	recombinant tissue Plasminogen Activator
Sav-HRP	Streptavidin Horseradish Peroxydase
SDS	Sodium Dodecyl Sulfate
SD	Standard Error
sec	seconds
SEM	Standard Error of the Mean
SNS	Sympathetic Nervous System
Stat3	Signal Transducer Activator of Transcription-3
SOCS	Suppressor Of Cytokine Signaling
TAT	Transactivator of Transcription
TCRβ	T Cell Receptor-beta
TIA	Transient Ischemic Attack
TMB	Tetramethylbenzidine
TNFα	Tumor Necrosis Factor-alpha
TTC	2,3,5-Triphenyltetrazolium Chloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
VCAM-1	Vascular Cell Adhesion molecule-1

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• Benakis C, Bonny C, Hirt L. JNK inhibition and inflammation after cerebral ischemia. *Brain Behav Immun* 24, 800-11 (2010).

ABSTRACTS

• Benakis C, Bonny C, Hirt L. The JNK signaling pathway and inflammation as a target for treatment in cerebral ischemia. Chicago, IL: Society for Neuroscience, 2009.

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1. INTRODUCTION

FIRST PART: CLINICAL AND CELLULAR ISSUE OF STROKE

1.1 STROKE EPIDEMIOLOGY AND CLINICAL IMPORTANCE

Stroke is a frequent and devastating disease. Up to one in five persons suffer from stroke during lifetime. Stroke is fatal for almost one-third of cases, making it the third cause of death in developed countries after coronary heart disease and cancer. Among those who survived, half are permanently disabled, making stroke a major cause of long-term physical, cognitive and social invalidity. The incidence of stroke is roughly equal in men and women, but increases dramatically with age. The absolute number of cases is likely to rise due to the aging of the population. There are several risk factors: men have a slightly greater risk of stroke particularly from the age of 45, family history of stroke, prior stroke or transient ischemic attack (TIA), high blood pressure, tobacco smoking, overweight, diabetes and heart condition. For the general population the warnings signs and symptoms of stroke might be difficult to detect because they are not exclusive to stroke. 'Stroke' was first named as 'stroke apoplexy' from the Greek word : *apo-plixia* which mean : a 'shock' that happens 'on, above'. Thus signs of stroke appear suddenly without warning. The main symptoms of stroke are: sudden numbness or weakness in one or both sides of the body, sudden difficulty to speak, to understand speech or confusion, sudden loss of vision, sudden vertigo, loss of coordination, amnesia and sudden severe headache with no known cause. Some symptoms like dysarthria or dizziness are not necessarily associated to stroke when there are isolated. Basically the diagnosis of stroke is made if the symptoms 1) are neuroanatomically localized; 2) correspond to a loss of function; 3) are of sudden onset; 4) evolve over minutes rather than over hours and days (Hankey, 2007; Williams, 2010).

1.2 ISCHEMIC STROKE

1.2.1 Acute management of ischemic stroke

Stroke is caused by arterial occlusion in 80% of all cases, while intracerebral hemorrhage and subarachnoid hemorrhage have a lower incidence. The more common thrombo-embolic stroke, which is discussed herein, appears when a blood clot occludes a cerebral artery and causes a focal interruption of cerebral blood flow. The vessel occlusion leads rapidly to impaired motor and sensory brain function such as hemiplegia, aphasia or hemineglect (see above). If the blood clot is not rapidly lysed within 3 to 4.5 hours with recombinant tissue plasminogen activator (rtPA) the neurological deficit can be irreversible and in severe cases lead to death (The NINDS & Stroke rt-PA Stroke Study Group, 1995; Furlan et al., 1999; Hacke et al., 2008). Indeed, when the blood flow drops, the brain tissue is affected in two steps : above a threshold neuronal function is compromised but recoverable which is described as the ischemic penumbra, but if the blood flow goes below this limit, irreversible neuronal damage occurs. Thus, early reperfusion could rescue the penumbral area and minimize brain damage. Figure 1 shows an ischemic stroke caused by the occlusion of the left middle cerebral artery and the favorable outcome induced by the thrombolytic agent.

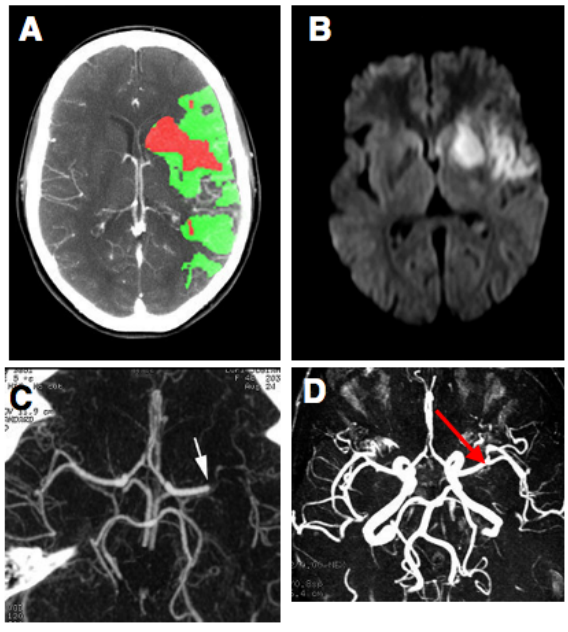


Figure 1. Ischemic stroke in the left middle cerebral artery

(A) Perfusion cerebral computerized tomography (CT) shows an ischemic core (red) surrounded by penumbra (green) (Wintermark et al., 2002). The core corresponds to the irreversible ischemic insult (infarction), whereas the penumbra represents brain parenchyma which is suffering from ischemia but which can be rescued if the blood perfusion is rapidly restored. (B) The diffusion weighted sequences on brain magnetic resonance imaging (MRI) show a hyperintensity in the territory of the left middle

cerebral artery. Of note, only the infarcted territory, which corresponds to the core in (A) is visible. (C) Angiographic sequences of cerebral CT show a sub-occlusion of the left middle cerebral artery (arrow). (D) The patient benefited from an intra-venous thrombolysis with rtPA. Few days later, angiographic sequences on brain MRI showed a complete repermeabilisation of the left middle cerebral artery, which paralleled a favorable clinical outcome (Benakis et al., 2009).

1.2.2 Other treatments

The thrombolysis is limited to a small number of patients, because of very strict selection criteria including a narrow time-window. Restoring the cerebral blood flow after a too long period from symptom onset is no longer beneficial and there is an increased risk of developing a symptomatic intracerebral hemorrhage (The NINDS & Stroke rt-PA Stroke Study Group, 1997). Other acute phase treatments are: administration of the intra-arterial of tPA or pro-urokinase directly into the occluded artery which extends the time window to six hours, and 'mechanical embolus removal in cerebral ischemia' (MERCI), a surgical technique that leads to the removal of the clot within 30 minutes. Both treatments are used in the acute phase and because of the need of advanced technology and specialized personnel, not all patients can benefit. If patients are not treated with rtPA they can receive aspirin in the acute phase to thin the blood and weeks after to avoid a subsequent ischemic stroke. Aspirin and other antiplatelet and anticoagulant drugs reduce the absolute risk of death and dependency several months after the episode, suggesting that it provides secondary prevention of a subsequent stroke rather than a direct protection. Other treatments are also focused on the prevention of a recurrent stroke, such as antihypertensive agents which aim at reducing the blood pressure or statins which reduce the cholesterol. A modification of the lifestyle and diet reduces the causal risk factors such as blood pressure, cholesterol and diabetes and therefore have an effect on reducing the risk of stroke. Surgical procedures are also used when a high grade carotid artery stenosis (artery not totally occluded) caused by atherosclerotic plaques leads to stroke (Haynes et al., 1994). An intervention is performed to remove the plaque and avoid an subsequent stroke. (Hankey, 2007; Williams, 2010).

Acute stroke treatments are very limited in time and technology. Other treatments listed above are mostly used to avoid recurrent stroke. As the damage extends with time there is a great need for new treatments for the patients who cannot be thrombolysed at first. Therefore new therapeutic strategies, which for instance target the delayed cellular and molecular mechanisms that follow cerebral ischemia, may be used with an extended therapeutic window for the protection of brain tissue.

1.3 CELLULAR AND MOLECULAR MECHANISMS FOLLOWING STROKE

Importantly, while ischemia at first induces a loss of function at its very early stages, structural damage appears rapidly thereafter and progresses as minutes and hours go by. Reduction of cerebral blood flow leads to a lack of oxygen and glucose supply to the brain parenchyma. This nutrient deficiency triggers multiple events including a dramatic depletion of energy (ATP) production, perturbation of the cellular ionic homeostasis, peri-infarct depolarization, water influx, neurotransmitter release and activation of many cytotoxic enzymes. Indeed the release of the excitatory neurotransmitter glutamate leads to excessive stimulation of glutamate receptors in energy-deprived neurons, termed excitotoxicity. As a result, the intracellular concentration of the secondary messenger calcium is dramatically increased and promotes numerous enzymes that degrade structural constituents of the cells as well as organelles such as mitochondria. Other proteases activate intracellular pathways or induce the production of free radical species, which activate apoptotic proteins, secretion of inflammatory mediators or damage the DNA. These mechanisms triggered by excitotoxic stimulation start in the early stages of the progression of the ischemic brain injury and can cause both acute cell death (necrosis) and delayed programmed cell death (apoptosis or autophagy) (Dirnagl, 1999; Lo et al., 2003; Puyal et al., 2009).

An important mechanism beginning within hours from the onset of ischemia is the robust inflammatory response in the ischemic tissue. During the acute phase of stroke, inflammation is a response leading either to restrict or to aggravate the progression of tissue damage. During the sub-acute phase, inflammation further induces

immunosuppression and favors opportunistic infections such as bronchopneumonia (Dirnagl et al., 2007). Figure 2 represents a schematic summary of the fundamental mechanisms taking place after focal cerebral ischemia: excitoxicity, oxidative and nitrosative stress, apoptotic-like cell death (Lo et al., 2003).

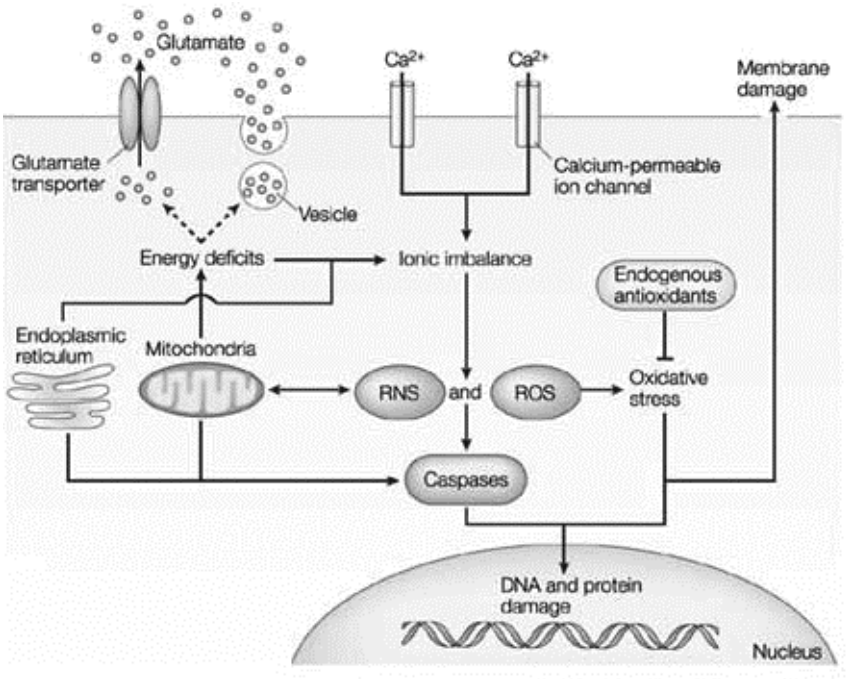


Figure 2. Major pathways implicated in ischemic cell death

Within minutes after cerebral ischemia excitotoxic mechanisms are activated in neurons in the territory of the occluded artery which lead to necrotic and apoptotic cell death. Ca^{2+} (calcium), RNS (reactive nitrosative species), ROS (reactive oxygen species). (Reprinted from Nature Reviews, 4, Lo et al., Mechanisms, challenges and opportunities in stroke, p400., Copyright (2003), with permission from nature publishing group).

1.4 NEUROPROTECTION TARGETING EXCITOTOXICITY

Excitotoxic mechanisms are activated within the first sixty minutes after cerebral ischemia. Thus, different strategies aimed at blocking these mechanisms have been developed for stroke therapy. These include inhibition of N-Methyl-D-Aspartic acid (NMDA) or Amino-Methyl-Propanoic acid (AMPA) receptors that are activated by glutamate, blockade of calcium channel, chelation of calcium, overexpression of anti-apoptotic components, etc [for review see: (Barone & Feuerstein, 1999; Lo et al., 2003;

Shuaib & Hussain, 2008)]. Most of these neuroprotection strategies are targeting very early mechanisms which might not be relevant in clinical therapy as most of the patients do not reach the emergency room early enough. Therefore inhibiting downstream events is likely to be more beneficial for neuroprotection.

1.4.1 The c-Jun N-terminal kinase pathway

Interesting candidates for stroke therapy are compounds that inhibit a single protein involved in deleterious events much downstream to the early cytotoxic effect. The Mitogen-Activated Protein Kinases (MAPKs) are a family of proteins kinases that respond to extracellular stimuli and regulate cellular signal pathways by cascades of phosphorylations. They consist of three enzymes activated in series: MAP kinase kinase kinase (MKKK, MEKK or MAP3K), MAP kinase kinase (MKK, MEK, or MAP2K) and MAP kinase. The p38 and the intracellular c-Jun NH_2-terminal kinases (JNK) are members of the MAPK family and are key signaling pathways activated by stress stimuli and further activate transcription factors that regulate cell survival, apoptosis and inflammatory cytokines production (Davis, 2000; Chang & Karin, 2001; Pearson et al., 2001). Indeed, Schwarzschild and colleagues have published that glutamate induces the activation of the JNK pathway (Schwarzschild et al., 1997). JNK activation triggers in turn the phosphorylation of several transcription factors including the nuclear c-Jun component of the transcription factor activator protein-1 (AP-1) and intracellular proteins as for instance members of the Bcl-2 family involved in apoptosis (Akins et al., 1996; Bogoyevitch et al., 2006). Evidence for JNK mediated c-Jun activation in apoptotic cells (TUNEL positive staining) was shown in animal models of transient focal ischemia (Herdegen et al., 1998). Therefore, c-Jun nuclear labeling is a hallmark of delayed neuronal death. Based on these findings, several therapeutic interventions aimed at inhibiting specifically the JNK pathway are under investigations.

JNKs activation is a complex mechanism that requires cascades of phosphorylation as in all MAPKs families. Ten isoforms resulting from alternatively spliced transcripts of the *jnk1*, *jnk2*, *jnk3* genes were identified in humans. JNK1 and JNK2 are expressed ubiquitinously whereas JNK3 is predominantly expressed in the nervous system. Thus,

the activation pathways of each JNK isoform might be cell and stimuli dependent but all require 1) three steps of consecutive kinase activation by phosphorylation and 2) specific localization. First, upstream to JNK activation, several different MEK kinases (MEKK1,2,3,4) will activate the second MAP kinase kinase : MKK4 and MKK7, both these kinases are the immediate activators of JNK. To be fully active JNK need to be phosphorylated on both threonine and tyrosine residues. Secondly, JNK interacting proteins (JIP) are scaffold proteins which bring together specific members of the JNK signaling pathway and allow their consecutive activation. When JNK is activated, it can interact with mitochondrial apoptosis-related proteins in the cytoplasm and mediate transmission of apoptotic signals (Okuno et al., 2004). Furthermore, JNK can be translocated into the nucleus and phosphorylates c-Jun on serine-63 and serine-73. This leads to the transcription of potent activators of apoptotic cell death such as those coding for Fas-ligand or Bim (Herdegen et al., 1998; Kyriakis & Avruch, 2001; Weston & Davis, 2007; Raivich, 2008).

1.4.2 Strategy of neuroprotection

Taking advantages of this tight regulation of the JNK pathway activation, small inhibitors were designed based on protein-protein interactions. To inhibit JNK, two strategies were used: 1) molecules that inhibit the ATP binding site of JNK which leads to inactivate the kinase (known as ATP-competitive inhibitors) or 2) small peptides that inhibit the interaction of the kinase with its targets (known as ATP-noncompetitive inhibitors) (Manning & Davis, 2003; Bogoyevitch, 2005a; Bogoyevitch et al., 2005b). The most used compound which inhibits the ATP binding site of JNK is the molecule SP600125 (Celgene Corporation). Several studies showed that SP600125 treatment prevented cell death following transient ischemia of the brain [as for instance (Gao et al., 2005)]. Despite the obvious efficacy of SP600125, some questions arose concerning its specificity for JNK. Indeed, these ATP-competitive inhibitors must discriminate between the ATP-binding sites of all protein kinases and must compete with high intracellular ATP concentrations. The second strategy using ATP-noncompetitive inhibitors was shown to be more efficient because the specificity was higher. Thus, small peptides of 10 to 30 amino acids derived from the JNK binding site

of the scaffold JIP were designed. To enhance their efficacy these peptides were linked to a cell-permeable sequence (HIV-TAT sequence) and to avoid its enzyme degradation D-amino-acids peptide were used instead of L-residues (Brugidou et al., 1995; Vivès et al., 1997; Haeusgen et al., 2009). A schematic cartoon of the action of such a peptide following stress stimuli is illustrated in Figure 3.

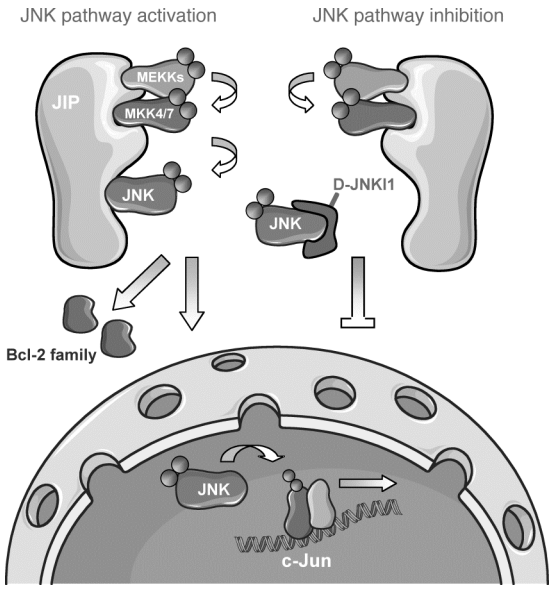


Figure 3. Inhbition of the JNK pathway using ATP-noncompetitive peptide

Consecutive activation of JNK by phosphorylation leads to its translocation into the nucleus where it can phosphorylate c-Jun which further leads to transcription of pro-apoptotic genes. The TAT-coupled peptide (here : D-JNKI1) binds specifically to JNK and inhibits its interaction with cytoplasmic and nuclear targets. (Figure was produced using Servier Medical Art).

1.4.3 Neuroprotective effect of D-JNKI1

As mentioned above D-JNKI1 is a selective ATP-noncompetitive inhibitor that prevents the interaction of JNK with its targets including c-Jun. Its sequence was first designed based on the JNK binding domain (JBD) of the islet brain-1/JNK-interacting protein (IB-1/JIP-1) in pancreatic beta-cells (Bonny et al., 2001). D-JNKI1 consists of a 10-amino-acid TAT-sequence, followed by two prolines and the 20 amino-acid JNK binding domain of the IB-1/JIP-1 protein in their D-configurations. This peptide shows a high degree of neuroprotection in several *in vitro* and *in vivo* models of cerebral ischemia (Borsello & Bonny, 2004). Indeed D-JNKI1 prevents NMDA-induced

neuronal death in primary cultures (Borsello et al., 2003; Centeno et al., 2007) as well as rescues the evoked potential response recorded in the CA1 region after oxygen and glucose deprivation (OGD) in organotypic hippocampal slice cultures (Hirt et al., 2004). Moreover, D-JNKI1 remarkably attenuates the lesion size in transient and permanent models of cerebral ischemia in both rats and mice (Borsello et al., 2003; Hirt et al., 2004; Repici et al., 2007; Esneault et al., 2008), even with delayed intravenous administration (Wiegler et al., 2008). These experimental studies have also shown that D-JNKI1 improves functional outcome at later time points after ischemia. All together, these data strongly suggest that the inhibition of the JNK pathway using D-JNKI1 would be very promising for stroke patients.

Several therapeutic strategies aimed at decreasing the effect of the ischemia-induced excitotoxicity have been successful in animal stroke models, but not so far in stroke patients. The efficacy of inhibitors of cell death would be greater if they target multiple mechanisms such as combined inhibition of excitotoxic events and of the detrimental effects of inflammation. The inflammation reaction is an attractive target for stroke therapy because of its rapid activation and progression within hours and days after stroke. However inflammation can have both favorable or unfavorable consequences on the ischemic lesion, it is fundamental to discuss its role in cerebral ischemia in order to better target drugs.

SECOND PART: INFLAMMATION DURING CEREBRAL ISCHEMIA

In cerebral ischemia, brain inflammation plays a role in the clearance of cell debris and initiates repair mechanisms; however, it can also enhance the damage to the tissue when the inflammatory response is not adapted or exacerbated. Thus, whether this brain inflammation is beneficial or detrimental in cerebral ischemia is still controversial. This short introduction gives insight on the key inflammatory players of this complex process.

1.5 ACTIVATION OF GLIAL AND INFLAMMATORY CELLS

The inflammatory response at the site of injury is characterized by the infiltration, accumulation and activation of glial cells and peripheral immune cells. Within hours from the onset of focal cerebral ischemia peripheral leukocytes adhere to the cerebral endothelium, cross the vessel wall and invade the damaged parenchyma (Petty & Wettstein, 2001; Wang et al., 2007). At the same time astrocytes and microglia become activated. These cellular events depend on the secretion of immunomodulators, which are produced by neuronal and glial cells in response to an ischemic insult. Once activated in the site of injury inflammatory cells start to secrete a large variety of inflammatory mediators such as cytokines, chemokines and promote the expression of adhesion molecules on the endothelial wall. Local brain inflammation is a pathological hallmark of ischemic stroke lesions and is related in space and in time to the occurrence of neuronal cell death (Barone & Feuerstein, 1999). Therefore, an assumption is often made regarding the possible role of inflammation in exacerbating the injury or its consequences.

1.5.1.Glial cells

Microglia are the resident macrophages of the brain, forming the 5-15% of the total cellular content of the brain. They are very sensitive to subtle alterations in their neuronal microenvironment. Microglia in their resting or rather in their ‘surveying’ state have a very ramified cytoplasm, covering a territory of 30-40 μm in diameter.

Their processes and ramifications are highly mobile which enable microglia to continually scan their environment. Thus, they can sense signs of slight functionnal and structural disturbance of the neighbors cells (Davis et al., 1994; Nimmerjahn et al., 2005; Hanisch & Kettenmann, 2007; Jinno et al., 2007). In response to an injury, microglia quickly become activated and undergo morphological transformations as well as functional changes. They start to retract their long processes and their shape becomes rounded - so called phagocytic, ameboid microglia (Raivich et al., 1999; Streit et al., 1999; Ladeby et al., 2005). When neurons die, microglia transform into brain macrophages and apoptotic cells are removed by phagocytosis. Figure 4 represents the different morphologies of microglia upon activation.

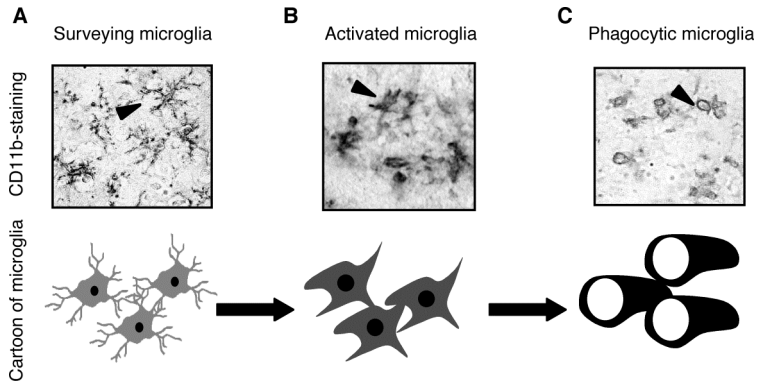


Figure 4. Activation of microglia after cerebral ischemia

Examples of immunohisto-chemical staining for CD11b, an integrin surface marker on microglial cells, after 48 h of reperfusion following 30 min middle cerebral artery occlusion (MCAO) in mice. Very ramified microglia (A, arrowhead) are detected in the cerebral hemisphere contralateral to the lesion. Activated microglia (B, arrowhead) and ameboid phagocytic microglia (C, arrowhead) are present in the injured brain tissue. Below is shown a cartoon of the graded activation of microglia.

Activated microglia were identified in patients with ischemic stroke within the territory of the middle cerebral artery (Gerhard et al., 2005; Thiel & Heiss, 2011). Gerhard and colleagues labeled microglia with a specific ligand for the translocator protein 18kDa (formely the peripheral benzodiazepine receptor PBR) present on activated microglia ([11C](R)-PK11195) (Stephenson et al., 1995). Using positron emission tomography (PET) scan they saw an increase of the marker in all the six patients examined between

3 and 150 days after the infarct. Figure 5 shows the lesion by MRI and the activation of microglia by PET scan for one patient at 5 and 13 days. Microglia are seen early after ischemia, initially at the periphery of the lesion (Figure 5e), then in the infarct core (Figure 5f) and finally in peri-infarct regions (Gerhard et al., 2005; Thiel & Heiss, 2011). This progression of microglial activation is not yet well understood.

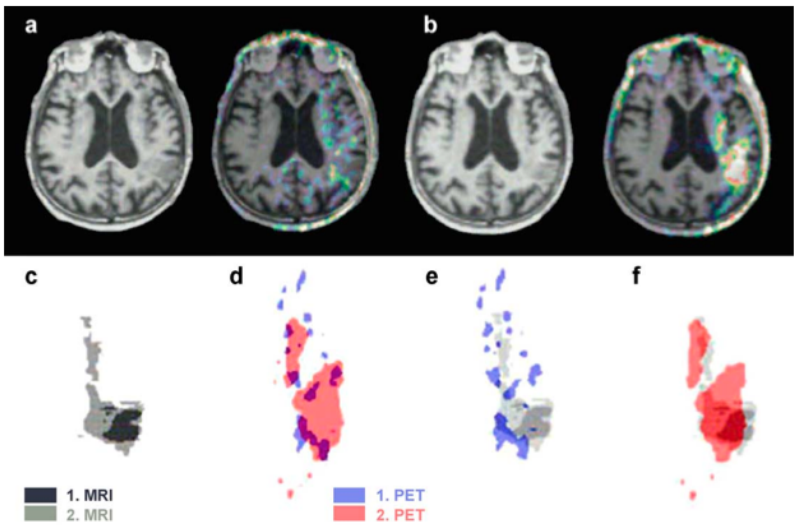


Figure 5. Activation of microglia in a patient after ischemic stroke

[11C](R)-PK11195 PET and MRI scans for patient 2 at different time points: Patient 2 was examined 5 (a) and 13 (b) days after the infarct. (e) and (f) illustrate the relationship between the increasing size of the post-ischemic lesion delineated by T1-weighted MRI hypointensity and the changes in the distribution pattern of increased [11C](R)-PK11195 binding after 5 (e) and 13 (f) days, respectively. (Reprinted from NeuroImage, 24, Gerhard et al., Evolution of microglial activation in patients after ischemic stroke: a [11C](R)-PK11195 PET study, p593., Copyright (2005), with permission from Elsevier).

These stimulated microglial cells rapidly proliferate to focal sites of injury due to an increasing expression of immunoreactive cell surface molecules and to the secretion of various pro- and anti- inflammatory molecules as well as chemotactic factors which induce the recruitment of other microglial cells. The morphological changes are accompanied by an increased expression of cytokines: interleukins (IL1β, IL4, IL6, IL10), tumor necrosis factor-α (TNFα), interferons and chemokines such as MCP1 and GROα (or KC) (Hanisch, 2002; Garden & Möller, 2006). The surrounding astrocytes

are sensitive to the increased release of these immunomodulatory molecules and undergo enlargement of cell bodies and thickening of processes as well. Activated astrocytes modulate in turn the phagocytotic functions of microglia, and promote the expression of adhesion molecules in the neurovascular unit on endothelial cells and circulating leukocytes. (Stoll et al., 1998). Glial cells secrete also different factors that are neuroprotective, like the nerve growth factor and brain-derived neurotrophic factor (BDNF) (Matsushima et al., 1998). On one hand, the release of these protective factors and inflammatory molecules may contribute to the rescue of damaged neurons and restrain the propagation of the injury, on the other hand the massive accumulation of inflammatory mediators and activated inflammatory cells may enhance the neuronal death.

1.5.2 Inflammatory cells

Shortly after the onset of injury the blood brain barrier opens by the disruption of the endothelial tight junctions (Zoppo & Hallenbeck, 2000). As mentioned above the release of inflammatory mediators from activated glial cells induces in turn the expression of proteins on the outer cell membrane of vascular endothelial cells. The opening of the barrier and the release of inflammatory mediators leads to the migration of circulating immune cells to the site of injury (Gelderblom et al., 2009). Infiltration of leukocytes (monocytes, neutrophils, lymphocytes) occurs in three steps: rolling on the surface of endothelial cells, adhesion to the endothelial wall and migration or diapedesis. The initial capture and migration is mediated by three main groups of cell adhesion molecules: selectins (P-, E- and L-selectins), immunoglobulins (VCAM-1, ICAM-1) and integrins (CD11a-c) (Petty & Wettstein, 2001; Sughrue et al., 2004). Activation and accumulation of leukocytes in the injured parenchyma results in the production of several agents in the ichemic tissue, such as reactive oxygen species (nitric oxide, superoxide) and metalloproteinases (Iadecola et al., 1999; Zoppo et al., 2000; Zhu et al., 2002). Their up-regulation triggers damage to the DNA and other cells constituants as well as the extracellular matrix and basal lamina (Dirnagl, 1999; Rosell et al., 2008). These factors potentiate neuronal death and may contribute to hemorrhagic complications after ischemic stroke. On the contrary, some of the agents

released might be protective, such as for instance nitric oxide which inhibits platelet aggregation (Iadecola, 1997).

Inflammation following cerebral ischemia differs by some aspects from inflammation of peripheral tissues. In the periphery, dendritic cells (DCs) participate to the immune response during local infection or other inflammatory situations. They are professional antigen-presenting cells which lead to the stimulation of T lymphocytes against pathogenic antigens (Steinman, 1991). The central nervous system was thought to be an immunologically privileged site and would not normally contain dendritic cells (Pedemonte et al., 2006). However, increased numbers of DCs in the ischemic parenchyma were observed in a permanent model of cerebral ischemia and the increase was correlated with the brain lesion area (Kostulas et al., 2002). In an other mouse experimental model of stroke, DCs were found in the border zone of the infarct and remote from the lesion and seemed to be associated with phagocytosis function (Reichmann et al., 2002). Thus tissue injury could lead to the activation of DCs in the parenchyma. Others by using labeled DCs transgenic mice found resident DCs in the healthy brain which increase in number and up-regulated the major histocompatibility complex II (MHC II) after transient experimental cerebral ischemia. Therefore they would be able to act as antigen-presenting cell and stimulate T cells (Felger et al., 2010). Thus, interestingly DCs could be resident immune cells of the brain and can be activated upon injury. This activation seems to be associated with phagocytosis and stimulation of T cells. Furthermore, Felger and colleagues showed that peripheral DCs could also infiltrate the ischemic parenchyma. Indeed due to the leakage of the blood brain barrier (BBB) after cerebral ischemia, cells from the periphery invade the parenchyma and it is postulated that by their capability to phagocytose dead cells, DCs would have immunogenic competence, activate T lymphocytes and enhance the inflammatory response in the brain tissue (Savill et al., 2002; Felger et al., 2010). However the function of DCs in the central nervous system (CNS) is not well characterized. Their role as immuno-competent cells and their contribution to damage require further investigations.

1.5.3 Ambivalent aspects of interleukin-6 (IL6) following ischemic stroke

Several clinical and experimental studies have found that cerebral ischemia promotes an important secretion of IL6 in the brain, in the cerebrospinal fluid (CSF) and in the systemic circulation. Indeed, the serum level of IL6 stroke patients was elevated early, within 24 h after the injury and sustained up to 5 to 7 days (Emsley et al., 2003). This increased concentration of IL6 was correlated with lesion size, early neurological impairment, body temperature and long-term poor outcome [for review see: (Suzuki et al., 2009; Denes et al., 2010a)]. However, Tarkowski and colleagues showed that only increased initial CSF level of IL6 and not the serum level was associated with infarct volume 2 to 3 months after stroke (Tarkowski et al., 1995). They also suggest that the main source of cerebral IL6 was the brain since IL6 level in the CSF was significantly higher than systemic level. Even though the exact origin of the peripheral IL6 is unknown, the fact that CSF concentrations are higher than serum concentrations at early time points strongly suggests that the source of IL6 is in the brain. Furthermore IL6 cellular localization in a context of experimental cerebral ischemia was principally in neurons as well as in glial cells (microglia and astrocytes) and endothelial cells (Suzuki et al., 2009). Additionnally, autopsy of a stroke patient showed IL6 positive neurons (Suzuki et al., 2002). Regarding the early increase of IL6 in the plasma and CSF and its correlation with infarct volume, IL6 was proposed as an early biomarker for ischemic lesion and poor outcome. However, it is not clear whether the production of IL6 in stroke patients may be the result and not the cause of enlarged cerebral infarction, and whether the IL6 increase is linked to other mechanisms such as physiological stress, previous inflammatory disease or vascular risk factors, or both (Zhou et al., 1993; Dziedzic et al., 2003). Instead, many experimental studies propose a non-detrimental effect of IL6 following experimental cerebral ischemia. A neuroprotective effect of exogenous IL6 has been observed in cultured cortical neurons exposed to NMDA-induced excitotoxic necrosis and *in vivo* 24 h after permanent MCAO (Loddick et al., 1998; Ali et al., 2000). However, in a model of transient MCAO of IL6 deficient mice, the infarct size and neurological function were not different from the control group (Clark et al., 2000). To clarify the endogenous role of IL6 mediated neuroprotection, an anti-mouse IL6 receptor monoclonal antibody (IL6-

ra) was administered to mice after MCAO (Yamashita et al., 2005). IL6 has been described to activate the intracellular Janus Kinase-Signal Transducer Activator of Transcription (JAK-STAT) pathway, which leads to the phosphorylation of Stat3 in neurons and subsequent modulation of expression of target genes. In normal conditions Stat3 is not phosphorylated, whereas a peak of activation is seen 24 h after MCAO (Suzuki et al., 2001). Yamashita and colleagues have shown that IL6-ra administration inhibits Stat3 phosphorylation and that was correlated with enlarged infarct volume. Furthermore, the majority of apopotic cells (caspase-3 positive) were p-Stat3 negative. Therefore, p-Stat3 activation mediated by IL6 might be associated with neuroprotection (Yamashita et al., 2005; Wang et al., 2009).

The role of IL6 release after cerebral ischemia remains complex and ambivalent. Suzuki and colleagues suggest that IL6 secretion might be time dependent as illustrated in Figure 6 (Suzuki et al., 2009).

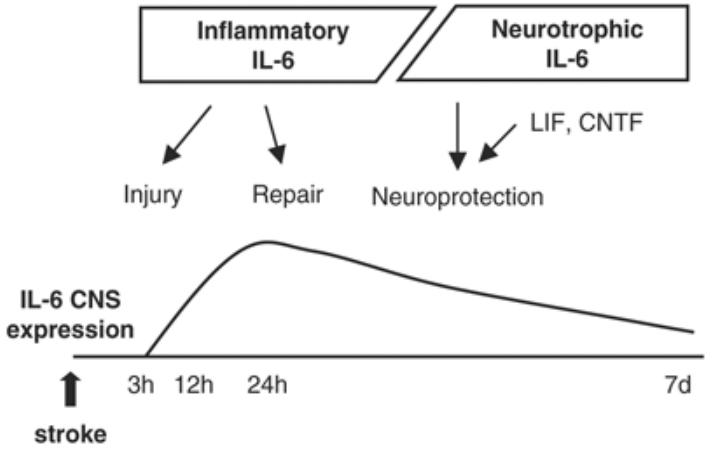


Figure 6. Ambivalent aspects of interleukin-6 after cerebral ischemia

In the CNS, IL6 works as an inflammatory cytokine during the acute phase of cerebral ischemia and may contribute to both injury and repair. However, the peak in IL6 expression is associated with neuroprotection in conjunction with leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF). (Reprinted from Journal of Cerebral Blood Flow & Metabolism 29, Suzuki et al., Ambivalent aspects of interleukin-6 in cerebral ischemia: inflammatory versus neurotrophic aspects, p474., Copyright (2009), with permission from nature publishing group).

1.5.4 Role of chemokines following ischemic stroke

Chemokines are highly up-regulated during cerebral ischemia. The CXC chemokines are capable of inducing migration and activation of neutrophils into inflammatory sites. An increase level of CXC chemokines such as CXCL1 (also named keratinocyte-derived chemokine KC or growth-regulated oncogene (GRO)/cytokine-induced neutrophil chemoattractant (CINC) in rodents which has the same function as IL8 in human) was found in the brain in pathological conditions. The major source of CXC chemokines upon brain injury are activated microglia, astrocytes, endothelial cells and infiltrated neutrophils, but not neurons [for review see (Semple et al., 2010)]. Transient cerebral ischemia in the rat correlated with an increase of CINC concentration first in the systemic circulation and then in the brain, preceeding brain neutrophilic infiltration (Yamasaki et al., 1995). Others have found the same spatial and temporal pattern of chemokine secretion and support the role of this chemokine leading to the potentiation of neutrophil-induced brain injury (McColl et al., 2007; Chapman et al., 2009). McColl and colleagues showed as well a reduction of the lesion volume and severity of the neurological deficit in neutrophil-depleted mice challenged to IL1β, suggesting that IL1β-mediated recruitement of neutrophil via CXC chemokines induction is detrimental for outcome after experimental stroke (McColl et al., 2007). Furthermore, repertaxin, an inhibitor for the receptor of the inflammatory chemokine CXC ligand 8 (CXCL8/IL8) was shown in transient cerebral ischemia to reduce neutrophilic infiltration, infarct volume, long-term reactive gliosis and microglial staining and, importantly, to improve the long-term outcome (Garau et al., 2005; Villa et al., 2007). Peripheral blood of patients with ischemic stroke was assessed for circulating IL8 and blood mononuclear cells expressing IL8 mRNA from day 1 to day 7 after onset, both were elevated compared to healthy patients (Kostulas et al., 1998). Systemic circulation of chemokines and their secretion in the ischemic parenchyma could lead to a concentration gradient over the BBB and would promote the influx of neutrophils into the damaged area.

1.6 IMMUNOSUPPRESSION FOLLOWING STROKE

Systemic inflammation is important in the context of stroke. Indeed there is clinical studies supporting the concept that systemic inflammation can be: 1) a risk factor for a stroke (McColl et al., 2009) and 2) a response to brain injury. It has been suggested that the latter could influence the outcome and long-term prognosis following stroke.

Bacterial pneumonia is the most frequent severe infectious complication occuring after stroke. It is estimated that 10% of deaths following admission for stroke are caused by pneumonia (Prass et al., 2006; Chamorro et al., 2007; Emsley & Hopkins, 2008). In an experimental model of cerebral ischemia Prass and colleagues showed that mice suffered from spontaneous bacterial infections within three days. The infection was accompanied with reduction of lymphocyte counts in the blood and the spleen already within a few hours after ischemia and lasting for several weeks (Prass et al., 2003). This phenomenon of brain-induced immunosuppression is also described in stroke patients with some evidence that these systemic changes occur before clinical manifestations of infection [for review see (Meisel et al., 2005; Emsley & Hopkins, 2008)]. This suggests that the peripheral immune system and the central nervous system are closely related, so that a brain injury causes a systemic immunological response. However, the mechanism by which the CNS injuries trigger immuno-supression remain unknown. One can assume that in response to the activation of numerous inflammatory cells and mediators in the ischemic tissue, the nervous system would counteract this response by the release of immunomodulators which downregulate the immune system. On the other hand, infections occuring after stroke might be a cause of adverse conditions of the patients as, for instance, immobilization, invasive procedures or dysphagia. Studies on the influence of post-stroke infection on clinical outcome have been disappointing due to confounding factors (Emsley & Hopkins, 2008).

There is evidence of systemic inflammation occurring after stroke but whether there is a direct link between these ‘supersystems’ (Tada, 1997): the nervous system and the

immune system, is still not well defined. Overall a better understanding of the relationship between the brain and the immune system might lead to more effective therapies in patients with acute stroke.

1.7 NEUROPROTECTION TARGETING INFLAMMATION

The general hypothesis that inflammation following cerebral ischemia could harm the brain and contribute to the progression of the damage comes initially from experimental studies focusing on the inhibition or reduction of post-stroke inflammation.

1.7.1 Targeting inflammatory cells and immunomodulators

Different strategies aimed at preventing the inflammatory response after cerebral ischemia have been successful in rodent models. Ischemic damage was shown to be attenuated: by inhibition of microglial activation using the minocycline tetracycline derivative, by systemic leukocyte depletion, by diminishing the free radical generating enzymes such as the inducible nitric oxyde synthase (iNOS) or the cyclooxygenase-2 (COX-2), by inhbition of cytokines secretion or by reduction of proteolytic degradation of the BBB [for review (Dirnagl, 1999; Iadecola & Alexander, 2001; Benakis et al., 2009].

1.7.2 Targeting inflammatory gene expression

The secretion of inflammatory mediators is tightly regulated by intracellular signaling pathways [for review see: (Kaminska, 2005)]. The strength and duration of the induction of inflammatory genes expression is essential to adapt correctly the intensity of the inflammatory response. Krause and colleagues showed that the JNK pathway, upon activation by a stress stimuli, namely IL1, induced the expression of IL6 and the chemokine IL8 (the human ortholog of CXCL1/KC) through the AP-1 complex in human keratinocyte cells (Krause et al., 1998; Holtmann et al., 1999). Thus modulation

of the JNK pathway could regulate the expression level of inflammatory mediators and might give insights on their function in cerebral ischemia.

Based on the hypothesis that inflammatory mediators enhance the damage of the ischemic tissue, several studies have found beneficial outcome by inhibiting gene expression of pro-inflammatory cytokines (del Zoppo et al. 2000). In reaction to stress signals p38 and JNK pathways positively regulate transcription of inflammatory genes, such as those coding for TNFα, IL1β, IL6, IL8 (Waetzig et al., 2005). The role of p38 and JNK on the regulation of transcription of these inflammatory mediators makes them interesting targets for anti-inflammatory therapeutic intervention [for review see (Barone & Feuerstein, 1999; Kaminska, 2005)]. The inhibition of these intracellular pathways activated upon injury leads to a reduction of inflammatory molecule production, as for instance using the upstream inhibitors of both JNK and p38 pathways: CEP1347, which reduces the production of cytokines after lipopolysaccharide (LPS) administration induced cytokine release in human and murine microglia, as well as after intracerebroventricular injection of LPS in mice (Lund et al., 2005). Using SP600125, a JNK inhibitor, Waetzig and colleagues showed that upon LPS stimulation of microglial cell cultures, there was a reduction of the induction of the AP-1 target genes: COX-2, TNFα, MCP1 and IL6 (Waetzig et al., 2005). However, Nijboer and colleagues showed that in a model of perinatal hypoxia-ischemia of rats, the inhibitor of JNK: L-JNKI1 was neuroprotective but did not reduce the expression of cytokines and chemokines (Nijboer et al., 2010). Because of the redundancy and overlap of inflammatory mediator function *in vivo*, it is uncertain that a specific inhibitor targeting a single site would have any detectable effect on the reduction of inflammatory molecules. Besides others have found opposite results using the mixed-lineage kinases inhibitor: CEP-11004, the release of cytokines was enhanced when JNK was inhibited in cultivated microglia (Hidding et al., 2002). Thus, the inhibition or activation of inflammatory gene expression might dependent of: the cellular type, the target of the inhibitors and their concentration, the model used (*in vitro* or *in vivo*) and the type of stimulation.

1.7.3 Bench to clinical trials

Unfortunately attempts to translate anti-inflammatory therapeutic interventions to stroke patients have been more disappointing than promising. For instance, the selective IL1 receptor antagonist, IL1-ra, which limits the pro-inflammatory action of IL1, has been tested in randomised patients with acute stroke (Emsley et al., 2005). Despite a conclusive phase II study no recent publications have reported the recombinant human IL1-ra as a therapeutic agent for acute stroke. More than a thousand potential agents underwent clinical evaluation (O'Collins et al., 2006). However none of these drugs have demonstrated benefit in stroke clinical trials as for instance the application of murine mono-clonal anti-ICAM-1 in the Enlimomab trial (Enlimomab Acute Stroke Trial Investigators, 2001) and NXY-059, a nitrone-based free radical trapping agent (Lees et al., 2006). Reasons for failure have been highlighted in the sections above and include morphological and functional differences between rodents and humans, timing of the intervention, ambivalent role of inflammatory mediators as well as evaluation of efficacy, pharmacokinetic issues (plasma concentration of drugs) and side effects [for review (Dirnagl, 2006)]. Anti-inflammatory strategies have also in some cases promoted deleterious infections and fever (Enlimomab Acute Stroke Trial Investigators, 2001).

Until now, researchers have focused mostly on the negative role of inflammation after stroke and thus have attempted to inhibit post-stroke inflammation. Nevertheless, there is evidence suggesting that inflammation might also be beneficial in stroke: it is a crucial mechanism to clear damaged tissue after an infarction, it promotes angiogenesis, tissue remodeling and regeneration (Dirnagl, 1999; Chamorro & Hallenbeck, 2006). Therefore, there is clearly a need to better understand the subtle balance between the beneficial and deleterious effects of inflammation in stroke. Furthermore, experimental studies on cerebral ischemia have mostly targeted one single cell type: neurons, while endothelium, astrocytes and microglia have been considerably neglected; or studies were focused on one single target rather than several targets as for instance different signaling pathways which are involved in the production of inflammatory mediators and apopototic proteins.

Future research on experimental stroke models should consider the important role of non-neuronal cells, the bivalent function of inflammation and probably the time dependency of post-stroke cellular and molecular mechanisms. A better insight in these aspects is important before planning future clinical trials.

AIMS OF THE PRESENT WORK

The aim of the present work was to explore the contribution of the JNK pathway in the modulation of neuro-inflammation using the specific inhibitor D-JNKI1 in experimental models of cerebral ischemia, in the purpose to better understand: 1) how D-JNKI leads to its neuroprotective effect and 2) the role of inflammation following stroke.

FIRST PART: EFFECT OF NEUROPROTECTION ON INFLAMMATORY CELLS

Our objective was to test whether D-JNKI1 can modulate the JNK pathway in microglia and if this contributed to the strong neuroprotective effect of delayed D-JNKI1 administration in our stroke models. First, we examined whether the activation of microglia was modulated in D-JNKI1-treated animals, subjected to transient MCAO in mice by investigating morphological changes and accumulation of microglia within the infarct area. Secondly, we looked at the cellular localization of D-JNKI1 and its effect on microglia in an *in vitro* model of cerebral ischemia.

SECOND PART: EFFECT OF NEUROPROTECTION ON THE SECRETION OF INFLAMMATORY MEDIATORS

Our second objective was to investigate the role of D-JNKI1 on the release of inflammatory mediators in the systemic circulation, peripheral organs and in the brain tissue following transient MCAO in mice.

2. METHODS

2.1 ANIMALS

Outbred male Crl:CD1/ICR mice (25-35 g, 4- to 5-week-old) and gestant Sprague-Dawley (OFA) rats were purchased from Charles River Laboratories Inc. (Charles River France, L'Abresle, France). All procedures were in accordance with the Swiss Law and were approved by the Swiss Federal Veterinary Office.

2.2 TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION (MCAO)

Focal cerebral ischemia was induced in CD1 mice using the filament method [see (Wiegler et al., 2008) and references therein]. Mice were anesthetized with isoflurane (induction: 3%, maintenance: 1.8% and reperfusion: 1.8% to 0%) in 70% nitrous oxide and 30% oxygen using a face mask. Body temperature was maintained at 37 ± 0.5°C throughout surgery (FHC Inc., Bowdoinham, ME, US). Regional cerebral blood flow (CBF) was continuously recorded by laser Doppler flowmetry (LDF, Periflux 5000, Perimed, Sweden) with a fiber optic probe fixed on the left lateral surface of the skull (1 mm posterior and 6 mm lateral from the bregma) during a period covering the induction of ischemia until 10 min after the end of ischemia. Transient cerebral ischemia (30 min, 45 min or 60 min) was induced by occlusion of the left middle cerebral artery (MCA). A silicone-coated nylon monofilament (diameter: 0.17 mm, Doccol Co., Redlands, CA, USA) was inserted through the common carotid artery and advanced into the internal carotid artery until mild resistance was felt and a decrease of CBF below 20% of the baseline indicated occlusion of the origin of the MCA. The left MCA was occluded for a given time and then the filament was withdrawn to allow reperfusion above 50% of the CBF baseline. Mice were given 0.025 mg/kg of buprenorphine subcutaneously for post-surgery analgesia and were housed in an incubator at 31°C for 24 h for recovery. Sham animals underwent the same procedure except for the MCA occlusion and were anesthetized during the same time as MCAO mice.

2.3 GROUPS, TREATMENT AND NEUROLOGICAL DEFICITS

2.3.1 Intra-venous administration of D-JNKI1

Randomly, saline solution used as a vehicle solution (NaCl 0.85% Medium, bioMérieux) or D-JNKI1 (0.1 mg/kg, manufactured by NeoMPS, obtained from Xigen SA, Epalinges, Switzerland) was injected intravenously (i.v.) into the tail vein of CD1 mice using a 1 ml syringe (Omnifix - 1 ml, Terumo needle 25G) and a mouse restrainer (Braintree Scientific Inc.) 3 h after ischemia onset. Sham mice received saline solution, or D-JNKI1 in a separate set of experiment.

2.3.2 Neurological deficit

The mice were sacrificed at different time points after MCAO. The lesion volume was quantified 48 h after cerebral ischemia. The neurological deficit was evaluated just before sacrifice. Neuroscore was graded for severity using the following scale: 0 = no observable neurological deficit, 1 = failure to extend the right forepaw, 1.5 = intermittent circling behavior, 2 = circling behavior, 3 = loss of walking or uncontrolled movement (Bederson et al., 1986; Hirt et al., 2004).

2.4 QUANTIFICATION OF NEURONAL DAMAGE

Mice were anesthetized with intra-peritoneal injection of 8 mg/kg xylazine (Rompun® 2%, Bayer) + 100 mg/kg ketamine (Ketanarkon 100, Streuli Pharma AG) and transcardially perfused with phosphate buffer solution (PBS) followed by 4% paraformaldehyde (PFA). In Figure 7, mice were sacrificed, brains were removed and directly frozen in liquid nitrogen vapor. Serial coronal 20-µm thick, 620-µm (perfused brain) or 720-µm (frozen brain) distant cryostat sections were used for lesion size measurements. Digitalized images of the Nissl stained tissue were acquired under a light stereomicroscope (Leica MZ16FA) and the ischemic area was measured by a blinded observer with ImageJ 1.38x software (NIH, http://rsb.info.nih.gov/ij/). The direct infarct volume was calculated by multiplying the sum of the infarct areas on each section by the distance between the sections. For a separate set of experiments (Figure 21), lesion size was evaluated qualitatively using 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining (Schilichting et al., 2004). Mice were sacrificed and

brains were removed, sliced in 2-mm-thick sections and one slice was stained with 2% TTC for 10 min at in the dark at room temperature (RT), followed by an overnight fixation with PFA.

2.5 ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES (OHC)

Three hundred and fifty micrometers thick coronal hippocampal slices from 10- to 12-day-old Sprague-Dawley rats were cultured on porous membrane units (Millicell-CM, Millipore, UK) in wells containing 1 ml of culture medium with 25% horse serum (Oxoid, UK), 50% minimal essential medium supplemented with HEPES and sodium bicarbonate (MEM, Gibco, UK), 25% Hanks' balanced salt solution (HBSS, Gibco, UK), 2 mM L-glutamine and 36 mM D-glucose. Cultures were grown at 33°C, 100% humidity and 5% CO_2. After 3 days the medium was replaced by fresh culture medium and, after 6 days, by a medium with 15% horse serum, 60% MEM, 25% HBSS, 2 mM L-glutamine and no glucose (Badaut et al., 2005).

2.6 OXYGEN AND GLUCOSE DEPRIVATION (OGD)

Hippocampal slice cultures were subjected to *in vitro* ischemia by exposure to reduced oxygen and glucose concentrations as described previously (Hirt et al., 2004). At day 8, slices were transferred to a serum-free hypoglycemic medium, DMEM (D5030; Sigma, USA) supplemented with 1 mM D-glucose and 2 mM L-glutamine, in a humidified hypoxia chamber (COY, USA) with a hypoxic atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 37°C for 30 min. Control cultures were placed at 37°C for 30 min, in a humid normoxic atmosphere. D-JNKI1 diluted in PBS (final concentration 12 nM) or an equal volume of PBS was added 6 h after OGD. Cell death was determined in the CA1 region using the fluorescent viability indicator propidium iodide (PI, final concentration 5 µg/ml, Sigma). PI fluorescence emission (excitation wavelength 568 nm) was measured 48 h after OGD and quantified densitometrically, using ImageJ software. After subtracting the background fluorescence, the results were expressed as a percentage of maximal cell death obtained by incubating the hippocampal slice cultures in PBS for 24 h at 4°C. Cell death was averaged for the four slices of each culture.

2.7 IMMUNO-STAINING

2.7.1 Staining of degenerating neurons: FluoroJade B

FluoroJade B staining (0.0004% FluoroJade® B, Millipore, UK) was performed on frozen sections according to the Chemicon International Company protocol. Sections were then mounted in Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

2.7.2 Immunohistochemistry staining for glial and inflammatory cells

Sections were blocked in 0.3% H_2O_2 for 20 min at RT followed by 10% normal goat serum (NGS, Invitrogen, Paisley, UK) in PBS with 1% bovine serum albumin (BSA) for 1 h at RT. Sections were incubated overnight at 4°C with the following primary antibodies: rat anti-CD11b (Mac-1, 1:100, AbD SEROTEC) or biotinylated Isolectin B4 (IB4, 1:500, *Griffonia simplicifolia* lectin I, Vector Laboratories) to label microglia, mouse anti-GFAP (1:500, Millipore) for astrocytes, hamster anti-TCRbeta (1:200, BioLegend) for T lymphocytes, hamster anti-CD11c (1:200, BioLegend) for dendritic cells. Using the immunoperoxidase method, biotinylated anti-rat (1:500, BA-9400, Vector Laboratories, Burlingame, CA), anti-mouse (1:500, Vector Laboratories, Burlingame, CA) or anti-hamster (1:500, Biolegend) IgG antibodies were applied for 1 h followed by avidin-biotin-horseradish peroxidase complex (Vectastain kit, Vector Laboratories, Burlingame, CA). Incubation with biotinylated secondary antibody was omitted for Isolectin B4. The immunocomplex was visualized by 3,3'-diaminobenzidine (DAB kit, Vector Laboratories, Burlingame, CA) and mounted with Eukitt.

2.7.3 Double immunofluorescence

After blocking in 10% NGS (±0.1% Triton X-100) sections were incubated overnight at 4°C in various combinations of the following primary antibodies: mouse anti-NeuN (1:500, Millipore), mouse anti-GFAP (1:500), biotinylated Isolectin-B4 (1:500) and rabbit anti-phospho-c-Jun(Ser63) or phospho-c-Jun(Ser73) (1:100, Cell Signaling). Antigens were visualized with the appropriate fluorochrome-conjugated secondary antibodies: alexa fluor 488 anti-rabbit (1:500, Invitrogen), alexa fluor 594 anti-mouse (1:500, Invitrogen), Cy3-conjugated Streptavidin (1:500, Jackson ImmunoResearch

Laboratories) for 1 h at RT. Sections were counterstained with DAPI (1:10,000, 1 mg/ml, SIGMA) and mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). Images were acquired with the Aviovision v3.1 software in a Zeiss Axiovision microscope. No specific immunoreactivity was observed in control sections prepared by omitting primary antibodies.

2.7.4 Immunofluorescence on OHC

OHC were fixed in 4% paraformaldehyde for 1 h at 4°C, blocked 1 h at RT in 10% NGS and incubated overnight at 4°C with the following primary antibodies: biotinylated IB4 (1:500), mouse anti-NeuN (1:500), rabbit anti-phospho-c-Jun(Ser63) or phospho-c-Jun(Ser73) (1:100) and rabbit anti-D-JNKI1 (1:300, Xigen SA). After rinsing in PBS, slices were incubated with Cy3-conjugated Streptavidin (1:5000), alexa fluor 594 anti-mouse (1:200) or alexa fluor 488 anti-rabbit (1:200) for 1 h at RT, mounted using Vectashield Mounting Medium and viewed under a Leica SP5 confocal laser-scanning microscope.

2.8 QUANTIFIACTION OF CD11B POSITIVE MICROGLIAL CELLS

The rat CD11b antigen was revealed using the secondary antibody IRDye 800-labeled anti-rat IgG (1:2,000, Rockland) for 1 h at RT. CD11b infra-red emission was measured at 800 nm with an Odyssey infrared imaging scanner (Li-COR, Biosciences, Lincoln, Nebraska, USA). The sections were counterstained with Nissl after the first scan to avoid detection of Nissl blue staining in the 800 nm channel, and rescanned at 700 nm for delimitation of the ischemic zones. The average intensity of microglia ($AvIntCD11b_{Total}$) was calculated within the ischemic region for each brain as follows: $AvIntCD11b_{Total} = (\Sigma_i RawIntensity/\Sigma_i pixels) - AvInt_{Background}$, with $i = i^{th}$ coronal section of a brain, $\Sigma_i RawIntensity/\Sigma_i pixels$ = AvIntCD11b without background correction and $AvInt_{Background}$ = average intensity of the background measured by omitting the primary antibody. One animal was excluded in the vehicle-treated group, because of unconvincing IRD-800 labelling.

2.9 PREPARATION OF SAMPLES FROM CD1 MICE FOR CYTOKINE/ CHEMOKINE RELEASE ASSAY

Plasma was obtained from anticoagulated (1/16 v/v of 4% Sodium Citrate) cardiac blood samples after centrifugation with 2,500 rpm for 15 min at 4°C. Control (no surgical procedure), MCAO or sham mice were transcardially perfused with PBS. Brain, spleen, liver and lung of mice were removed for organs culture. Two millimeters thick coronal brain slices and a fifty square milimeter fragments of spleen, liver and lung were incubated in wells containing 1.5 ml of DMEM culture medium (Dulbecco's Modified Eagle Medium 1X, 4.5 g/L Glucose, L-Glutamine, GIBCO) with 10% horse serum (Oxoid, UK) and supplemented with 11.0 mg/ml Sodium Pyruvate (100 mM, SIGMA) and 10 ml/L Penicilin : Streptomycin (SIGMA). Organs were incubated for 20 h in a cell culture incubator at 37°C with 5% CO_2 humidified air. The next day the liquid supernatants were removed and centrifuged for 5 min at room temperature (20°C) with 12,000 rpm. The supernatants were collected and frozen at -80°C. Plasma and supernatants samples were stored at -80°C until use for Enzyme-linked Immunosorbent Assay (ELISA).

2.10 PROTEIN EXTRACT FROM ORGAN CULTURES

Brain slices and fragments of spleen, liver and lung were homogenised in buffer (62.5 mM Tris-HCl pH 6.8, 50 mM DTT, 0.3% SDS) containing protease inhibitors. Each fragment was first chopped with scissors and then sonicated for 10 sec. After centrifugation (10,000 rpm, 15 min, 4°C), supernatants were collected and protein concentrations was calculated according to the Bradford method (Bradford, 1976).

2.11 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) ON PLASMA AND ORGANS CULTURE SUPERNATANTS

Plasma and culture supernatants from brain, spleen, liver and lung of control, sham (vehicle or D-JNKI1) and MCAO (vehicle or D-JNKI1) mice were tested by ELISA for detection of the mouse Interleukin-6 (IL6, OptEIA TM Set, BD Biosciences) and the mouse Keratinocyte Chemoattractant protein (CXCL1/KC, DuoSet ELISA

Development System). Ninety six-well plates were coated with 100 µl anti-mouse IL6 (70 ng/ml, 1/500) or KC (360 mg/ml, 1/360) capture antibody in 0.1 M Sodium Carbonate or PBS coating buffer. Plates were incubated at 4°C over-night, washed three times with buffer (1X PBS, 0.01% Tween 20) and blocked with assay diluent (1X PBS + 10% FCS or 1% BSA) for 1 h at RT. Plates were then washed three times and 50 µl of sample (1/3 for plasma and 1/5 for supernatant samples) or standard was added to each well. After 2 h incubation at RT, plates were washed three times. IL6 plates were incubated with 50 µl of the working detector solution (1/250 Detection Ab, 1/250 Sav-HRP) 1 h in the dark while KC plates were first incubated in 50 µl of KC detection Ab (1/160) for 2 h at RT, followed by a wash step and then 50 µl of the diluted Sav-HRP (1/200) was added for 20 min at RT in the dark. IL6 and KC plates were then washed five times. This was followed by addition of 50 µl TMB chromogen (DAKO, DiaLine). The color was allowed to develop for 15-20 min, and the reaction stopped by adding 50 µl stop solution (1 M H_3PO_4). Optical density was then measured at 450 nm.

2.12 STATISTICAL ANALYSIS

Results were expressed as mean ± SD (lesion volume) or SEM (inflammatory mediators release), except for neurological scores which were expressed as [median, min, max]. For comparing two groups, Student's t-test (two-tailed) was used (GraphPad Prism software 5.0). Analysis of correlation were done using the SAS software package. Normalization test was performed to check for significant violations in the distribution assumptions. For the analysis of the cytokine/chemokine concentrations in the second part of the results, were transformed into square-roots to obtain a normal distribution. Comparison of three or more groups was done by one-way or two-way ANOVA. For post-hoc comparisons, a Brown-Forsythe test for equality of variances between groups was first performed, followed by Tukey comparisons of means when variances were equal or Games–Howell test when variances were unequal (JMP Version 9.0.0 and IBM SPSS statistics Version 19.0.0). For analysis of survival difference between groups, the product limit method of Kaplan and Meier was used, and comparison of survival curves using the Log-Rank test. P values < 0.05 were considered significant.

3. RESULTS

FIRST PART: EFFECT OF NEUROPROTECTION ON INFLAMMATORY CELLS

3.1 EVALUTAION OF THE INFARCT VOLUME 48 H AFTER TRANSIENT MCAO UNDER ISOFLURANE ANESTHESIA IN CD1/ICR MICE

Because of toxicity issues, our lab replaced halothane anesthesia by isoflurane. Several durations of ischemia were tested under isoflurane anesthesia in order to match the lesion volume to our previously published data under halothane anesthesia (Clarkson, 2007; Wiegler et al., 2008). We hypothesized that the longer the ischemia lasts, the more we would obtain necrotic cell death and the less the anti-apoptotic peptide D-JNKI1 would have an effect on reducing the lesion size. Similarly, if the mice are less exposed to ischemia, the lesion volume would be smaller and the efficiency of the treatment would be harder to detect. Mice were subjected to ischemia for 30 min, 45 min or 60 min. The direct infarct volume was quantified on Nissl stained brain sections 48 h after the beginning of the occlusion. Figure 7 shows the direct lesion volume after 30 min MCAO ($V = 15.7 \pm 7.0$ mm^3, $n = 7$), 45 min ($V = 39.6 \pm 14.8$ mm^3, $n = 4$) and 60 min ($V = 77.9 \pm 6.9$ mm^3, $n = 4$). As we found a smaller lesion volume than previous experiments for the same duration of ischemia (30 min: 62 ± 14.8 mm^3, Wiegler et al 2008), we chose to adapt the time of ischemia. Accordingly, MCAO was performed for 30 min (Figure 8, Figure 11 and Second part) or 45 min (First part).

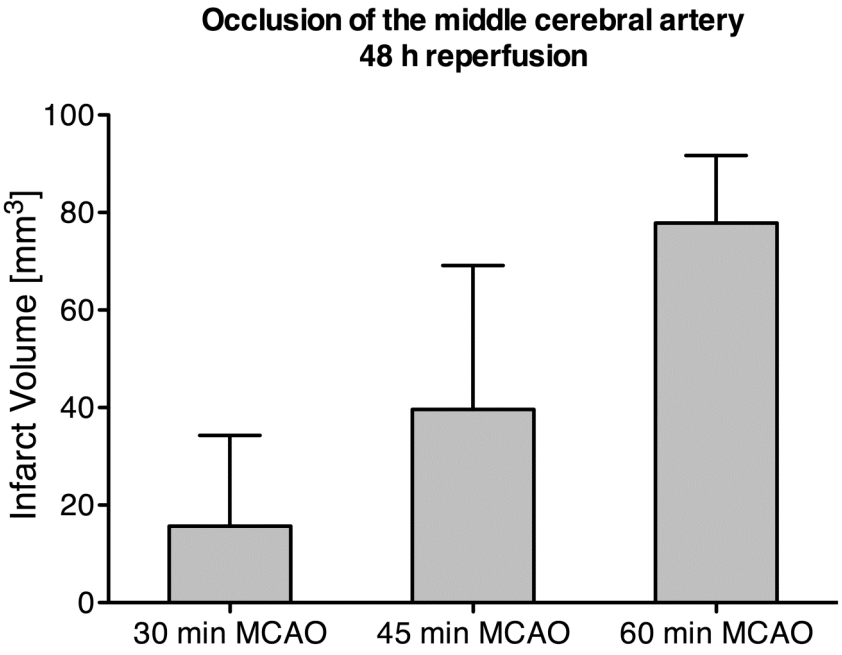


Figure 7. Infarct volume after transient MCAO

Mice were sacrificed 48 h after MCAO, brains were removed and directly frozen in liquid nitrogen vapour. Quantification of the direct infarct volume on Nissl stained coronal brain sections after 30 min, 45 min and 60 min MCAO shows a bigger infarct as a function of the duration of the occlusion: 15.7 mm^3 (30 min) < 39.6 mm^3 (45 min) < 77.9 mm^3 (60 min). Results are presented as mean ± SD, *n = 4 to 7.*

3.2 TRANSIENT MCAO INDUCES AN ACCUMULATION OF INFLAMMATORY CELLS IN THE ISCHEMIC TISSUE AT 48 H

In order to evaluate the effect of D-JNKI1 on inflammation, we investigated the activation and accumulation of glial and inflammatory cells after focal cerebral ischemia in mice by immunohistochemistry. We first characterized microglial activation at different time points after ischemia onset (9 h, 24 h, 48 h and 7 days). In our MCAO model, we found a peak of CD11b positive microglial cells in the ischemic tissue at 48 h (Figure 8). At earlier time-points, there was either no detectable CD11b-staining (9 h), or microglia were in a ramified state with a less dense staining (24 h). At 48 h, there were numerous microglia in the infarct area with an ameboid morphology. Seven days after MCAO, highly ramified microglia were present only in the injured

hippocampal tissue, whereas no positive staining was seen in the striatal ischemic region. Further experiments with microglia were performed 48 h after surgery when CD11b staining was most prominent.

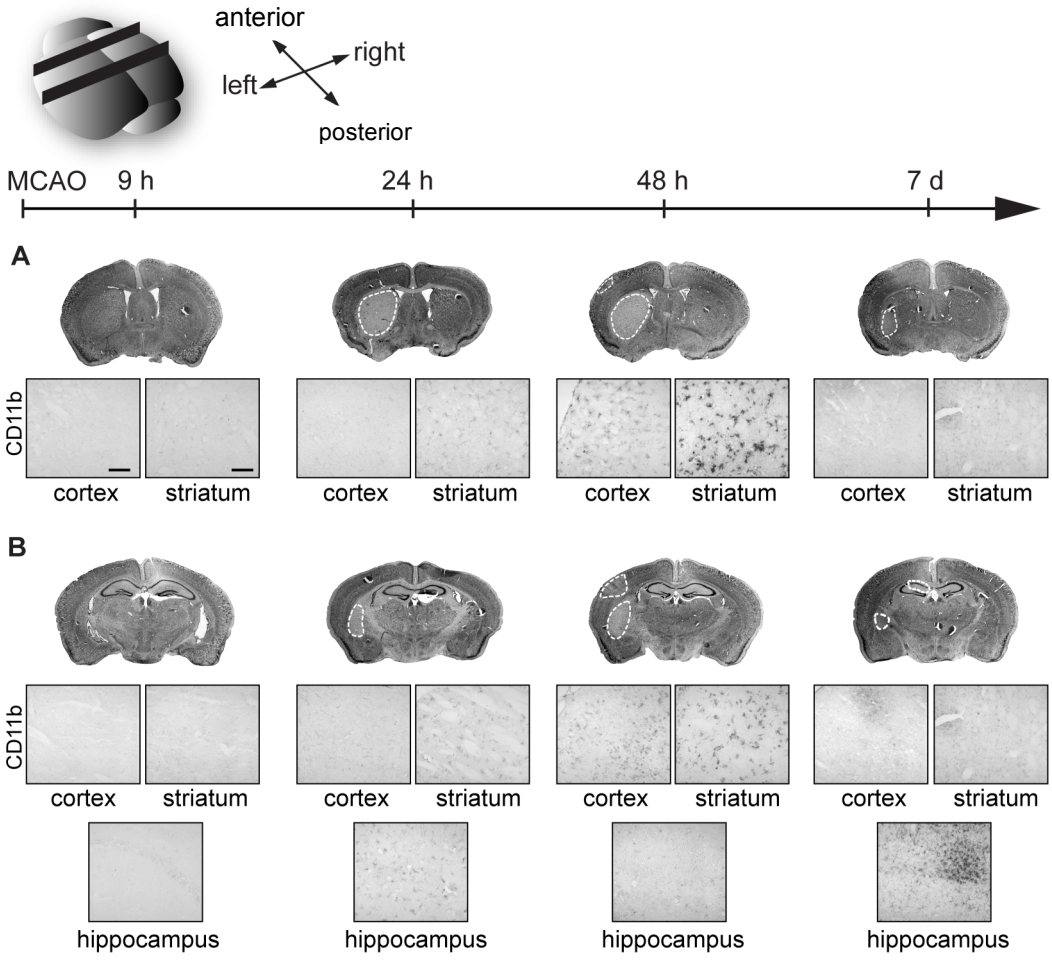


Figure 8. Time course of microglial activation after MCAO

The presence of CD11b microglial cells was analyzed by immunohistochemistry in the ipsilateral (ischemic) hemispheres at 9 h, 24 h, 48 h and 7 d after 30 min MCAO in vehicle mice. Two distant slices are shown (A: frontal cortex and anterior commissure, B: parietal cortex and hippocampus). Their location is indicated in the representative 3D scheme of a mice brain. Bar scale: 100µm.

At 48 h after 45 min transient MCAO, the infarct areas were localized in the striatal and cortical regions of the left hemisphere (Figure 9A, left) highlighted by a specific marker for degenerating neurons (FluoroJade B, Figure 9B). The cellular changes resulting in reactive astrocytes are an important component of glial activation after ischemia. It has been previously described that the function of reactive astrocytes is to create a physical barrier between damaged and healthy cells (Anderson et al., 2003). In our model of MCAO, we found that glial fibrillary astrocytic protein positive cells (GFAP+) with a stellate morphology were restricted to the infarct border, outside the ischemic zone, while hypertrophic GFAP+ cells were found within the ischemic tissue, suggesting a reactive gliosis 48 h after MCAO (Figure 9C). Activated microglia, characterized by an increase in size of the cell body and a decrease of ramifications, were highly present in the ischemic tissue as shown by Isolectin B4 and CD11b positive staining (Figure 9D and 9E). It has been postulated that dendritic cells (DCs) are activated in the ischemic tissue and have a phagocytic function (Reichmann et al., 2002). Staining for DCs using CD11c was found in the ischemic region (Figure 9F). Activation of microglia, dendritic cells and restriction of the extent of damaged tissue by astrocytes after stroke as well as the breakdown of the blood brain barrier could promote the infiltration and accumulation of T lymphocytes within the infarcted area (Raivich et al., 1996; Ishikawa et al., 2004). We performed immunohistochemistry using an antibody against the beta chain of the T cell receptor (TCRβ) and found positively stained T lymphocytes especially in the ischemic striatum (Figure 9G1).

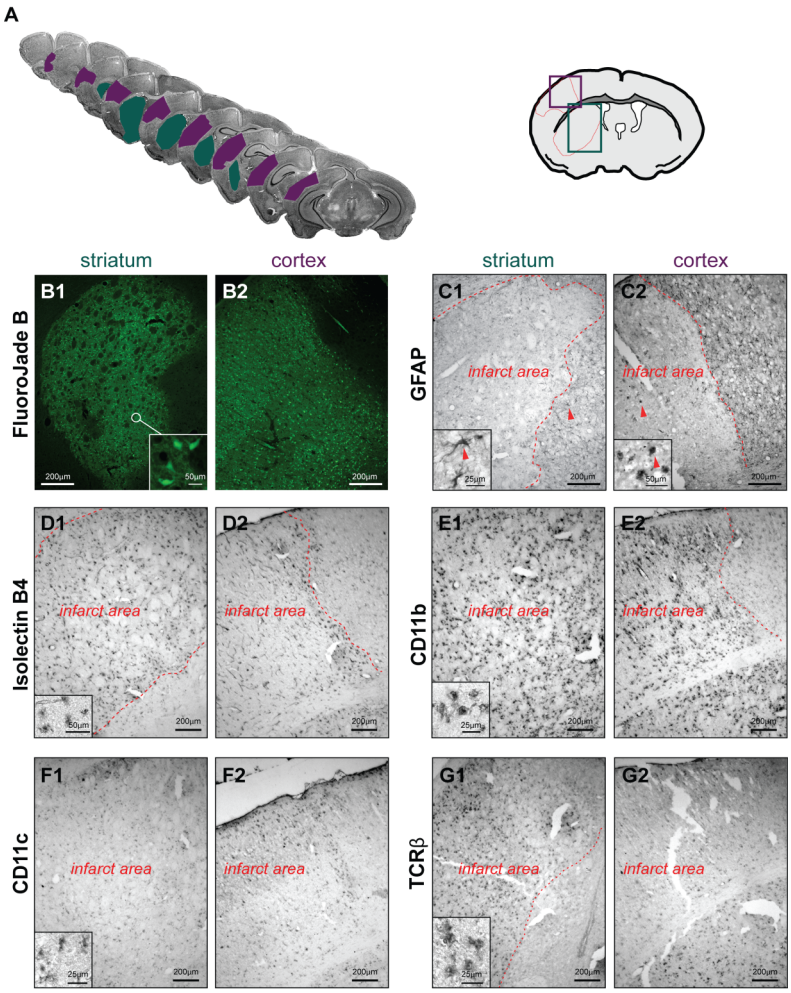


Figure 9. Glial and inflammatory cells in the ischemic tissue

CD1 mice were subjected to 45 min MCAO and brain sections immunostained 48 h after surgery. (A) Representative Nissl-stained coronal sections of the total infarct area 48 h after MCAO (left). Striatal and cortical damaged regions of the ipsilateral hemisphere are represented in blue-green and purple, respectively. The location of striatal (blue-green square) and cortical (purple square) ischemic areas where all immunohistochemistry data were analyzed are showed in the upper right panel. (B) Representative histological section 48 h after MCAO showing degenerating neurons (FluoroJade B) located within striatal (B1) and cortical (B2) ischemic lesions in the territory of the left MCA. (C) Reactive astrocytes (GFAP positive cells) are predominantly located in the spared parenchyma bordering the ischemic zone (C1, arrowhead), whereas hypertrophic astrocytes are within the demarcated area (C2, arrowhead). (D and E) A robust inflammatory response shown by activated (Isolectin B4 and CD11b positive) microglial cells is restricted to the infarct area clearly separated from immediately adjacent viable neural tissue. Inserts show high magnification of typical activated microglia.

(F and G) Dendritic cells (CD11c) and T lymphocytes (TCRβ), especially in the striatum (G1) are also detected within the ischemic area and not in the healthy contralateral parenchyma (data not shown).

Our data showed marked inflammatory changes 48 h after transient MCAO with a high degree of activation of glial cells, with reactive astrocytes surrounding the lesion and an accumulation of microglia within the ischemic tissue as well as the presence of inflammatory cells such as DCs and T cells.

3.3 MCAO INDUCES C-JUN PHOSPHORYLATION IN NEURONS AND GLIAL CELLS

Activation of the JNK pathway after ischemia is well described, resulting in the phosphorylation of JNK targets, including transcription factors in the nucleus such as the immediate early gene c-jun (Borsello & Bonny, 2004; Gao et al., 2005; Repici et al., 2007). Using specific antibodies directed against the phosphorylated serine-63 or -73 residues of c-Jun, we found phospho-c-Jun(Ser73) in cortical region as early as 9 h after MCAO (Figure 10) even though we could not detect marked tissue damage, by Nissl staining (see Figure 8). When the lesion was formed, activation of c-Jun was detected essentially in the cortical infarcted areas 24 h after MCAO, while at 48 h positive staining was in both cortical and striatal regions. As late as 7 days after MCAO induction we still observed p-c-Jun labeling in the ischemic hemisphere. The same time course was observed using the specific phospho-c-Jun(Ser63) antibody (data not shown).

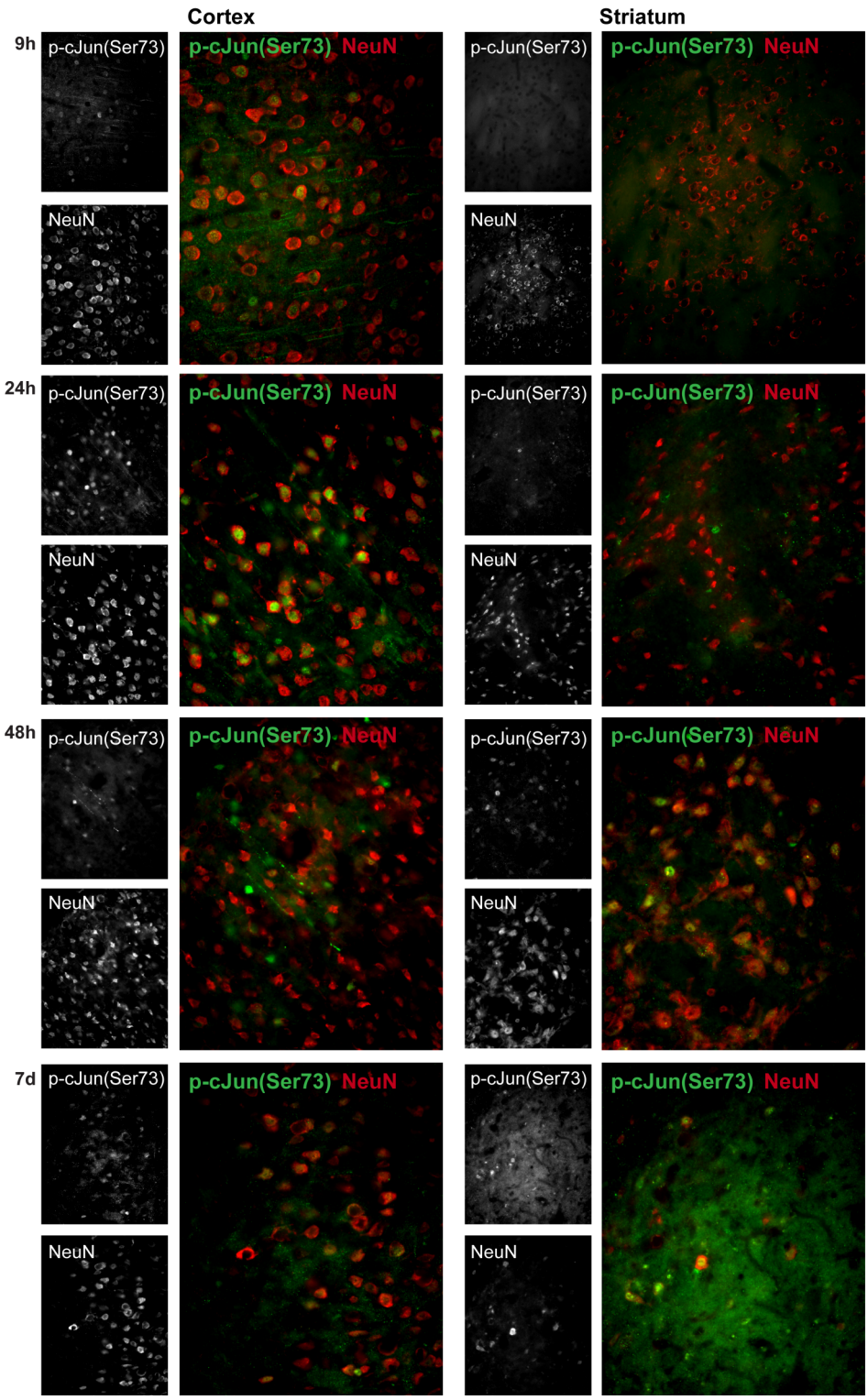


Figure 10. Time course of phospho-c-Jun activation after MCAO

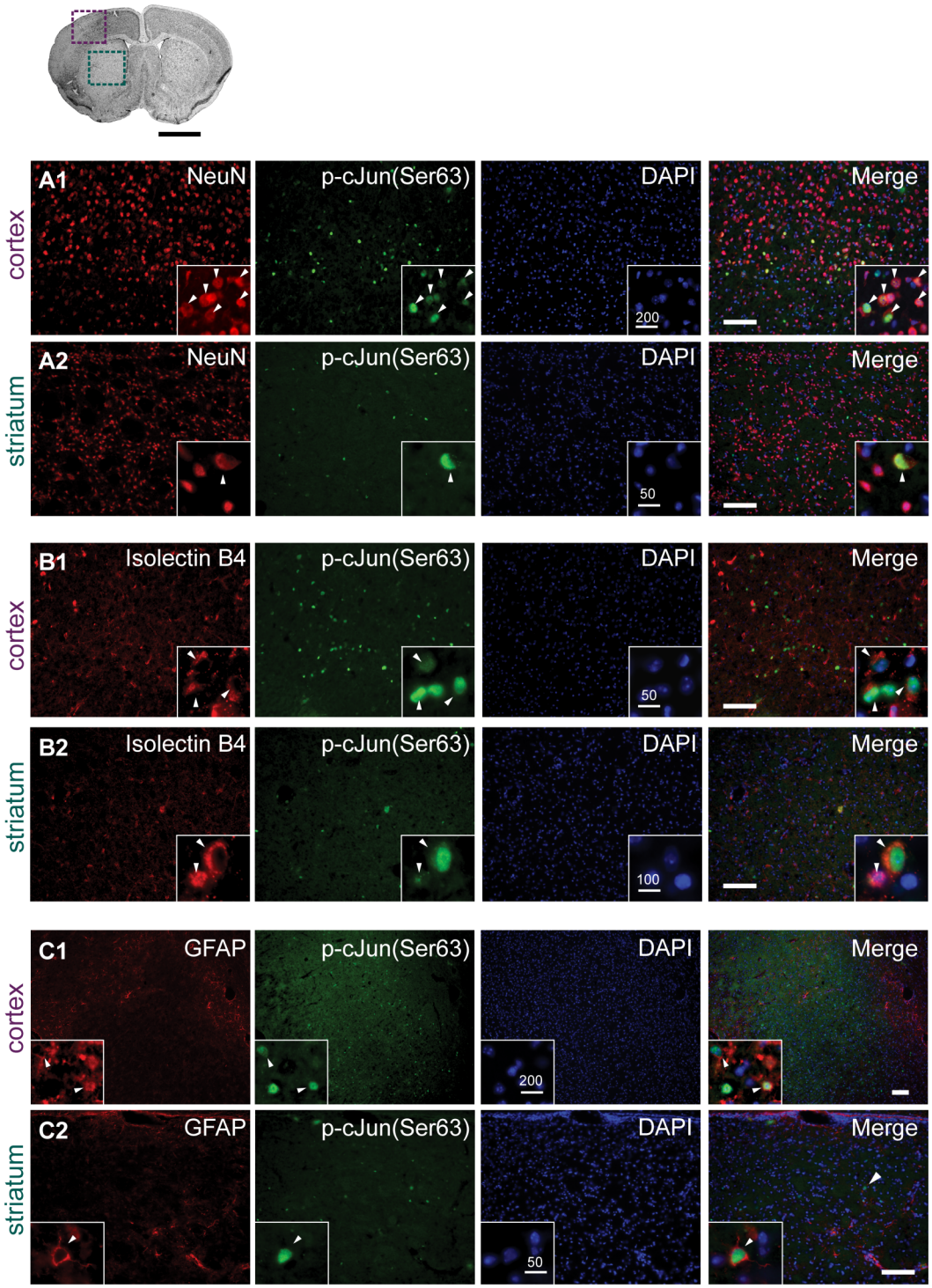
Double immunofluorescence for p-c-Jun(Ser73) (green) and NeuN (red) was performed at 9 h, 24 h, 48 h and 7 d after 30 min MCAO in vehicle mice. Photomicrographs show activation of c-Jun in the striatal and cortical ipsilateral (ischemic) regions.

In order to further characterize the role of the JNK inhibitor D-JNKI1, not only in neurons but also in glial cells, we performed double-labelling of p-c-Jun(Ser63) using different cell-specific markers as NeuN (neurons), Isolectin B4 (microglia) and GFAP (astrocytes) within the ischemic tissue 48 h after MCAO (Figure 11).

Despite early activation of c-Jun at 9 and 24 h, we chose to analyze a possible effect of D-JNKI1 at 48 h after ischemia onset, because we detected a peak of microglial activation (see Figure 8) and c-Jun is activated in the entire ischemic tissue at this time point (see Figure 10). p-c-Jun(Ser63) (green) was detected as expected in the nucleus of neurons, located predominantly in the cortex (red preserved shape, Figure 5A1, arrowheads), while in the striatal ischemic region we found apparently degenerating neurons and rare co-localizations with p-c-Jun (Figure 11A2, arrowheads). We also found a high degree of co-localization of microglia with p-c-Jun (Figure 11B1–B2, arrowheads). Higher magnification shows a nuclear localization of p-c-Jun in microglial cells in an activated ameboid-phagocytic macrophage-like morphology. p-c-Jun was also detected in GFAP+ cells in the cortical and striatal ischemic areas (Fig. 11C, arrowheads).

Figure 11. Activation of c-Jun in neurons and glial cells

The boxes on the brain section in the upper left show the striatal (blue-green) and cortical (purple) ischemic areas where immunofluorescence experiments were carried out. (A–C) MCAO promotes nuclear phosphorylation of c-Jun(Ser63) (p-c-Jun) (lines A1–C1). (A and B) Double fluorescent immunolabelling experiments show that p-c-Jun was highly detected in neurons (NeuN) (A1 and A2, arrow heads) as well as in activated microglia (Isolectin B4, B1 and B2, arrow heads). (C) Cytoplasmic GFAP positive cells merged with p-c-Jun are found in the striatal and cortical ischemic areas (C1 and C2, arrowheads). Inserts show high magnification of double labelling. Scale bar: 100 µm.



Taken together, these data demonstrate that c-Jun phosphorylation occurs in the ischemic areas (Fig. 11, boxes on the coronal brain section in the upper left) and is detected in neurons as well as in glial cells 48 h after MCAO.

3.4 THE HIGHLY NEUROPROTECTIVE JNK INHIBITOR D-JNKI1 DOES NOT REDUCE THE ACCUMULATION OF MICROGLIA IN THE ISCHEMIC TISSUE 48 H AFTER MCAO

To analyze whether D-JNKI1 has an inhibitory function on the activation and accumulation of microglia at 48 h, we quantified microglial cells after MCAO in treated and vehicle groups (D-JNKI1 or saline solution). First, mice were subjected to 45 min MCAO and treated i.v. with a single dose of 0.1mg/kg D-JNKI1 or saline solution 3 h after MCAO onset. 48 h after surgery, the lesion area could be clearly outlined by Nissl staining (Figure 12A, red line in microphotographs above the diagram). The direct lesion volume was 28.2 ± 8.5 mm^3 in vehicle mice (n = 7), and 13.9 ± 6.2 mm^3 in the D-JNKI1-treated mice (n = 6), with a significant 51% reduction of the lesion size ($p < 0.01$), confirming our previous findings (Wiegler et al., 2008). Figure 12B illustrates a typical Doppler curve measuring the cerebral blood flow from the beginning of the surgery until 10 min after reperfusion.

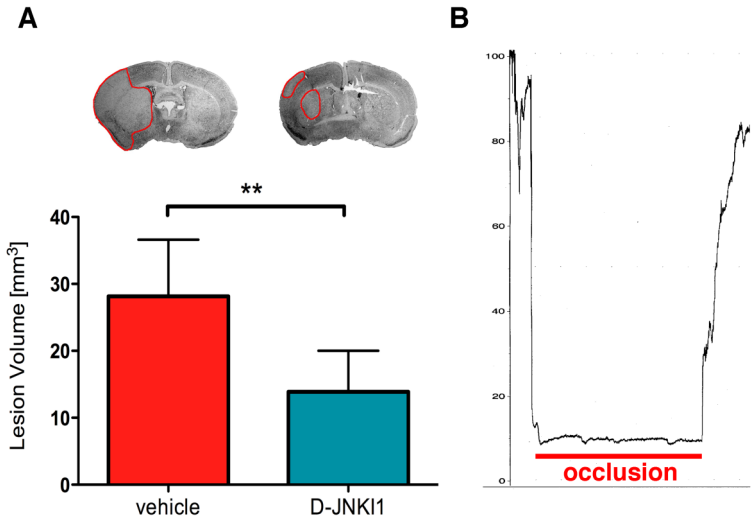


Figure 12. Neuroprotection with D-JNKI1 3 h after transient MCAO

(A) Intravenous administration of 0.1 mg/kg D-JNKI1 3 h after 45 min MCAO significantly reduced the infarct volume compared to vehicle-injected mice. Nissl-stained coronal sections show typical examples of lesion (**$p < 0.01$). Results are presented as mean ± SD, *n = 6 to 7*. (B) Representative diagram of the cerebral blood flow (CBF) as a function of time. The CBF was monitored from the beginning of the surgery until 10 min after withdrawal of the filament to follow the reperfusion.

Regional cerebral blood flow was measured by laser Doppler in all animals and is shown in Table 1. Data indicate satisfactory reduction in laser Doppler flow during ischemia in all animals and satisfactory reperfusion, slightly better in the D-JNKI1-treated group ($p = 0.033$). This minor difference in level of reperfusion does not explain the important reduction in lesion volume (Wiegler et al., 2008). Table 1 also shows similar weight and temperature measurements during surgery in both groups. Temperature at sacrifice was slightly lower in vehicle-animals than in D-JNKI1-treated mice ($p = 0.048$), which correlated with the reduction of the infarct volume in the D-JNKI1-treated group. There was no difference in weight between groups 48 h after the operation, probably due to the high distribution level of the lesion size in this MCAO model. Neuroscore scaled from 0 (best functional outcome, no deficit) to 3 (worst outcome) was not significantly better in the D-JNKI1-group (1.0, 0, 2.0) than in vehicle-group (1.0, 1.0, 2.0); $p = 0.701$, most likely due to the lack of sensitivity of this test. We have shown previously a beneficial effect of D-JNKI1 on functional outcome after MCAO using more sensitive tests (Borsello et al., 2003; Hirt et al., 2004; Wiegler et al., 2008).

Table 1

Measurements of CBF, weight, temperature and neuroscore in the group vehicle or treated with D-JNKI1. There was a slightly better reperfusion in the D-JNKI1 treated group ($p = 0.033$). Temperature at sacrifice was slightly lower in vehicle- than in D-JNKI1-treated mice ($p = 0.048$). Results are represented as mean ± SEM, except for neurological scores which are expressed as [median, min, max]. *$p < 0.05$ indicates a significantly difference between groups.

	Vehicle (n = 7)	D-JNKI1 (n = 6)
CBF during ischemia (%)	15.4 ± 2.1	13.2 ± 1.5
CBF after ischemia (%)	50.7 ± 2.3	63.0 ± 4.9*
Weight (g)	25.1 ± 0.9	25.0 ± 0.7
Weight at sacrifice (g)	19.6 ± 1.7	18.5 ± 1.0
T during ischemia (°C)	37.0 ± 0.1	36.7 ± 0.1
T at sacrifice (°C)	35.4 ± 0.2	36.3 ± 0.3*
Neuroscore at 48 h	1.0, 1.0, 2.0	1.0, 0, 2.0

In order to investigate the effect of D-JNKI1 treatment on the activation of microglia, we performed immunohistochemistry using a CD11b-specific antibody in sections from all animals 48 h after surgery. In a blinded study we could not identify morphologic changes (activated-ameboid microglia versus resting-ramified cells) between animals and groups. Representative CD11b immunostainings in the ischemic striatum of one animal per group are shown in Figure 13A. This suggest that D-JNKI1 does not influence changes in microglial shape in our MCAO model. We then quantified the number of microglial cells by measuring the average intensity of CD11b (Figure 13B, green) in the total ischemic area (Figure 13B, red staining, white line) in vehicle mice (n = 6) and D-JNKI1-treated mice (n = 6) 48 h after MCAO. Quantification analysis of CD11b average intensity did not show a significant difference between the vehicle and D-JNKI1 group (Figure 13C), suggesting that D-JNKI1 does not prevent the accumulation of microglia in the ischemic tissue. Figure 13D shows the correlation between the expression of CD11b and the brain lesion volume in both groups. As no modification of microglial activation was detectable after D-JNKI1 treatment, we hypothesized that there was a direct link between the activation of microglia and the size of the lesion independently of the treatment. There was a strong linear correlation between CD11b and lesion volume in the vehicle group (red square, $p < 0.01$). In the D-JNKI1 treated group, this correlation was negative and interestingly was not significant (blue green square) as it was in the vehicle group. However linear regression between groups showed that there was no significant difference.

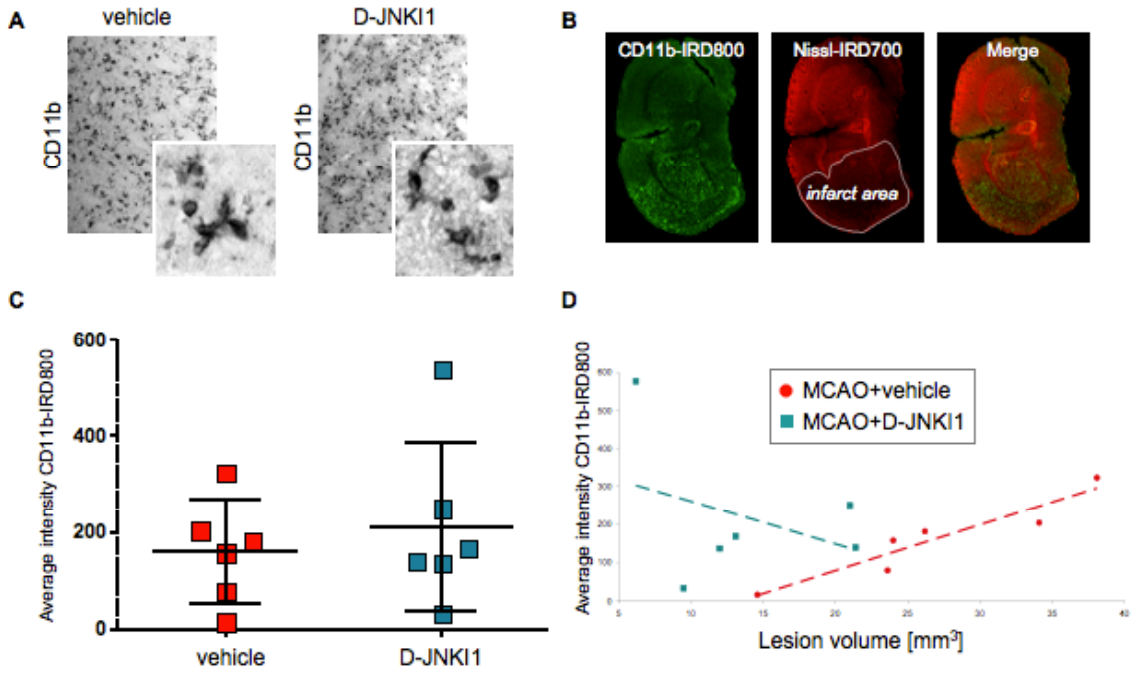


Figure 13. Quantification of microglia in the ischemic region in treated and vehicle groups

(A) Representative CD11b immunohistochemistry of vehicle- and D-JNKI1-treated animals. Inserts show high magnification of CD11b+ microglia. (B) Representative CD11b infrared emission staining (green) and Nissl-stained section (red) 48 h after MCAO. The measurements were done in the outlined ischemic area (white area). Merged scanned microphotograph shows that CD11b staining is localized within the ischemic tissue. (C) Quantification of CD11b average intensity in vehicle- (n = 6) and D-JNKI1-treated (n = 6) animals in the ischemic tissue. (D) Correlation between infarct volume and the average intensity of CD11b in D-JNKI1 (blue-green square) and vehicle groups (red square). There is a correlation between the infarct volume and the average intensity in vehicle-treated group (R = 0.94, p = 0.005). This correlation is negative and non-significant in the D-JNKI1-treated group (R = -0.36, NS). A linear regression analysis of slopes of D-JNKI1 and vehicle groups gives a non-significant difference between groups (R = -0.01, NS). NS, not significant. Results are represented as mean ± SD, *n = 6*.

These data showed that in our stroke model, D-JNKI1 strongly reduces the infarct volume but has no effect on the morphology and accumulation of microglia within the damaged tissue 48 h after MCAO. This suggests that neuroprotection with D-JNKI1 could occur independently of microglial activation in our cerebral ischemia model.

3.5 D-JNKI1 IS PRESENT MAINLY IN NEURONS 48 H AFTER OGD

The finding that MCAO activates p-c-Jun in microglia and that D-JNKI1 fails to inhibit the activation and accumulation of microglia despite a strong neuroprotective effect prompted us to investigate the cellular localization of D-JNKI1. The peptide was not detectable in the brain after i.v. injection of 0.1 mg/kg. Co-localization experiments were therefore assessed in our *in vitro* model of cerebral ischemia as depicted schematically in Figure 14A. First, we exposed organotypic hippocampal slice cultures to OGD for 30 min and treated cultures 6 h after OGD with 12 nmol/l of D-JNKI1. We measured PI uptake as an indicator of cellular death in the CA1 region of hippocampal slices in control conditions (CTRL), 48 h after OGD and after OGD followed by D-JNKI1 treatment. Figure 14B shows percent of cell death in the CA1 region in control slices (6.6% ± 1.8, n = 20), after OGD (28.5% ± 4.2, n = 39) and after OGD+D-JNKI1 (6.0% ± 2.1, n = 9). The neuroprotective peptide significantly reduced the PI uptake compared to slices subjected to OGD, indicating a significant 79% reduction of neuronal death ($p < 0.01$) confirming previous findings (Hirt et al., 2004). We then investigated the cellular localization of D-JNKI1 in these slices. We performed double-labelling 48 h after OGD on treated slices using antibodies against D-JNKI1 (green) and neurons (NeuN, red) or microglia (Isolectin B4, red) (Figure 14C). D-JNKI1 was found in the neuronal CA1 region with a cytoplasmic staining (squares), and a few positive cells were localized outside the CA1 neuronal layer (circles). Co-localization of D-JNKI1 was observed mainly in CA1 neurons (Figure 14C1, arrow heads) and in few microglia outside or at the border of the CA1 region (Figure 14C2, arrowheads).

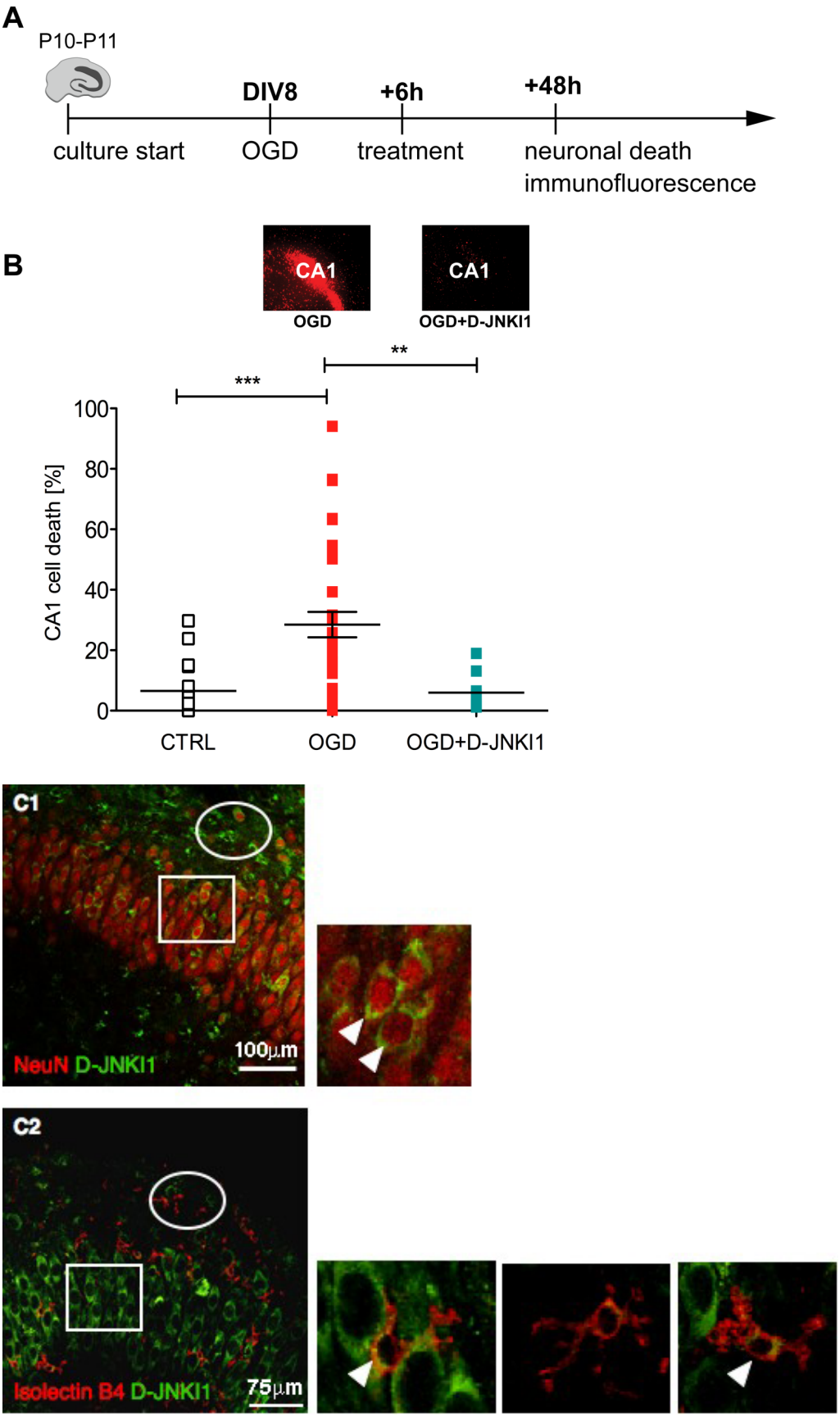

A
P10-P11
DIV8
+6h
+48h
culture start
OGD
treatment
neuronal death
immunofluorescence
B
CA1
CA1
OGD
OGD+D-JNKI1
**
***
100
80
60
40
20
0
CA1 cell death [%]
CTRL
OGD
OGD+D-JNKI1
C1
NeuN D-JNKI1
100µm
C2
Isolectin B4 D-JNKI1
75µm

Figure 14. Co-localization of D-JNKI1 in neurons and microglial cells 48 h after OGD in hippocampal slices

(A) Schematic representation of the experimental protocol. OGD for 30 min was performed 8 days after *in vitro* culture (DIV), 12 nmol/l D-JNKI1 or saline solution was added into the culture medium 6 h after OGD. Neuronal death and immunofluorescence were analyzed 48 h after induction of ischemia. (B) Effect of D-JNKI1 on neuronal death assessed by cellular PI uptake. Representative photomicrographs showing PI uptake in the CA1 layer of hippocampal slice cultures not exposed (left) and exposed (right) to vehicle or D-JNKI1, respectively. A significant decrease of PI uptake was observed 48 h after D-JNKI1 treatment compared to not treated slices subjected to OGD. The diagram shows percent of cell death in the CA1 region (CTRL versus OGD: $p < 0.001$; OGD versus OGD+D-JNKI1: $p < 0.01$), mean ± SEM, *n = 9 to 39*. (C) D-JNKI1 is mainly localized in the CA1 pyramidal cell layer of hippocampal slices 48 h after OGD and shows a cytoplasmic staining (outlined by white squares), whereas few stainings are localized outside the CA1 region (white circles). Co-localization of D-JNKI1 (green cytoplasmic staining) with NeuN (red nuclear labelling) is found in treated slices, predominantly in the CA1 (C1, magnification: arrowheads). Little co-localization was observed between D-JNKI1 and Isolectin B4-positive microglial cells at the border of the CA1 layer (C2, magnification arrowheads).

Taken together, these observations showed that D-JNKI1 is localized in the cytoplasm of CA1 neurons where it is likely to inhibit the JNK death signaling pathway, and that little co-localization of the peptide is observed in microglia, suggesting that D-JNKI1 could modulate the JNK pathway also in these cells.

3.6 D-JNKI1 MODULATES THE ACTIVATION OF MICROGLIA AFTER OGD

We have demonstrated that D-JNKI1 was neuroprotective *in vitro* and was found mainly in neurons as well as some microglia. To verify the molecular function of the peptide in these cells, we examined whether D-JNKI1 could inhibit c-Jun phosphorylation and modulate the activation of microglia. First by Nissl staining we observed that the CA1 layer is well preserved in control slices (Figure 15A1) and after D-JNKI1 treatment in slices subjected to OGD (Figure 15A3), whereas neurons after OGD without treatment show the typical shape of dying cells with their pyknotic nuclei (Figure 15A2). The same results were obtained using the nuclear specific antibody for

neuron NeuN (Figure 15B). Fluorescent double immunolabelling using NeuN and p-c-Jun(Ser63) showed, as expected, that in control slices there was no p-c-Jun staining in the neuronal layer (Figure 15B1). After OGD, the level of p-c-Jun clearly increased in the nuclei of neurons (Figure 15B2), whereas in treated slices fewer positive p-c-Jun were observed (Figure 15B3). Using the antibody against p-c-Jun(Ser73) we found a fibrillary staining in control and OGD+D-JNKI1 slices whereas after OGD p-c-Jun(Ser73) was activated in the nucleus (Figure 15C, green staining), accordingly with published data using the same antibody (Price et al., 2010). Surprisingly, we observed no colocalization of p-c-Jun(Ser73) or -(Ser63) in microglia after OGD as well as in D-JNKI1-treated slices subjected to OGD (Figure 15C and Figure 16).

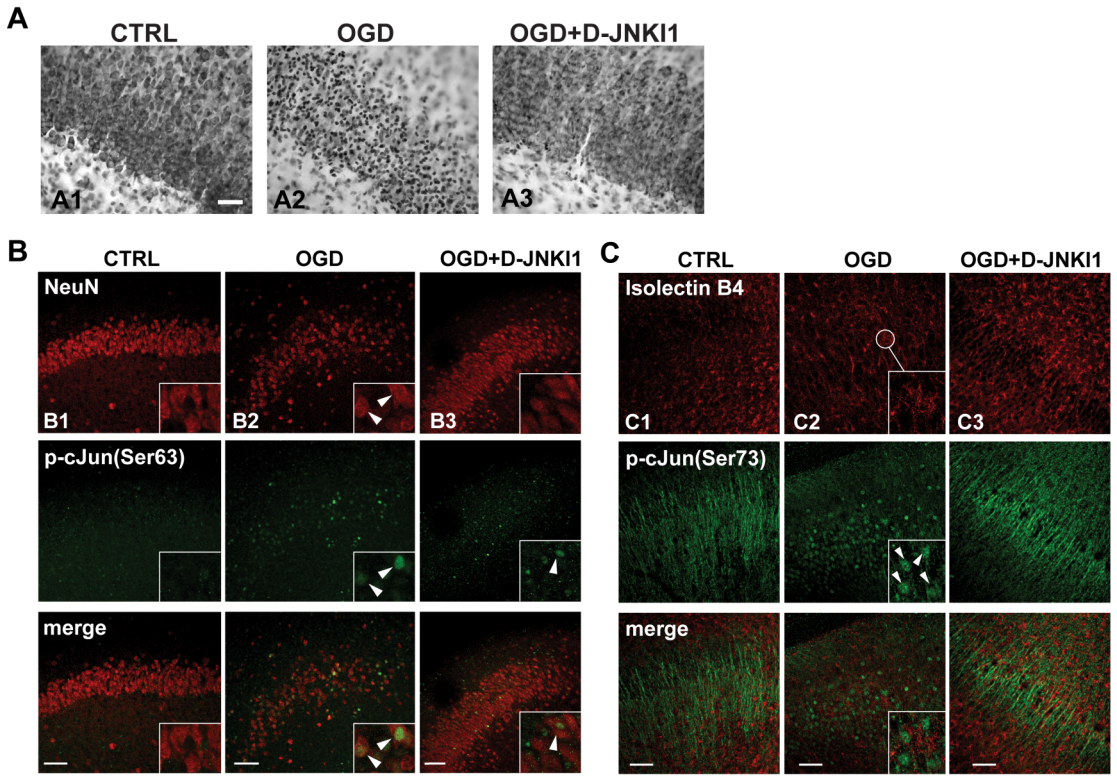


Figure 15. Activation of c-Jun after OGD

(A) Nissl-staining of the CA1 region of control (CTRL), OGD and OGD+D-JNKI1 slices. CA1 neurons are undergoing ischemic cellular death, shown by pyknotic nuclei (A2), whereas treated slices (A3) as well as control slices (A1) show neurons in a healthy state; scale bar: 50 µm. (B) After OGD nuclear expression of p-c-Jun(Ser63) co-localized with NeuN-positive cells (arrows), while NeuN staining is partially lost (B2) compared to control slices (B1). D-JNKI1 (12

nmol/l) treatment rescues NeuN staining while it partially attenuates p-c-Jun staining (B3). In both OGD (C2) and OGD+D-JNKI1 (C3) groups, no IB4 cells co-localized with p-c-Jun(Ser73) (arrowheads). Inserts show high magnification. All fluorescent microphotographs of double labelling are from the CA1 hippocampal region. Scale bar: 100 µm.

However, when we analyzed qualitatively microglial morphology *in vitro*, in control slices, we observed microglia with long thin processes, the typical structure of so-called resting or surveying microglia, homogeneously distributed throughout the slice (Figure 16A1-B1), while slices subjected to OGD showed microglia with less ramified processes and a round cell body (ameboid microglia) concentrated within the neuronal CA1 region (Figure 16A2-B2). In Figure 16A3-B3, D-JNKI1-treated slices show more ramified cells not specifically localized in the CA1 layer contrary to OGD slices.

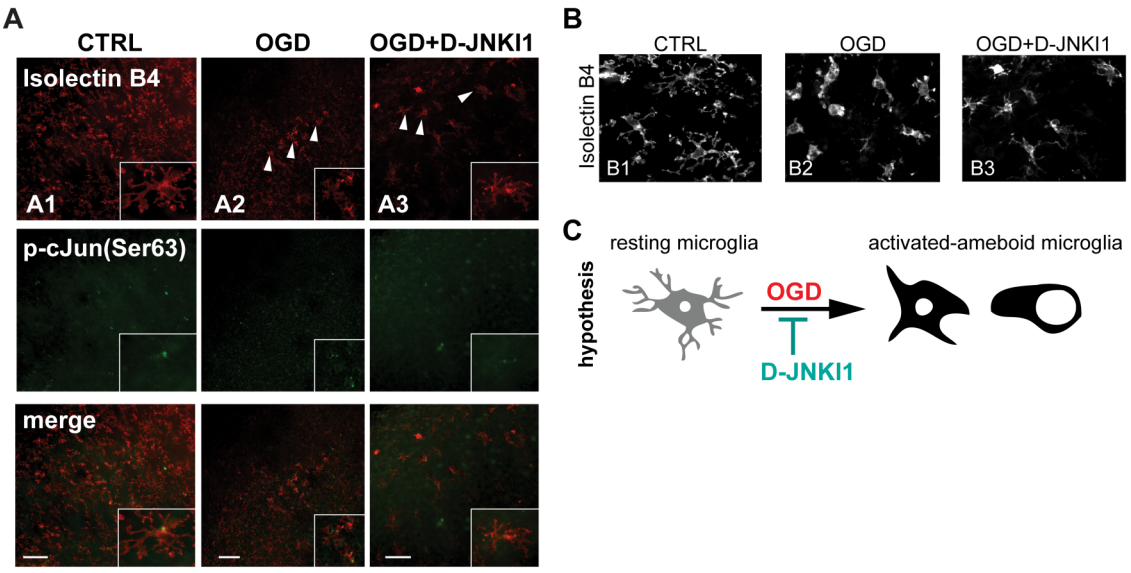


Figure 16. Effect of D-JNKI1 on microglia activation

(A-B) In control slices microglial cells are highly ramified and are evenly distributed (A1, red and B1). After OGD microglia accumulates in the damaged CA1 layer and changes its morphology to an ameboid shape (A2, arrowheads and B2), whereas in treated slices, morphology of microglial cells are more similar to ramified cells (A3, insert and B3), and are localized at the border or within the neuronal CA1 layer. Inserts show high magnification. (C) Schematic presentation of microglial morphological transformation in response to an ischemic insult such as OGD *in vitro*. Our initial hypothesis is that after an insult resting microglia become highly activated, further accumulate and could change morphology to a phagocytic state. We suggest

that D-JNKI1 may inhibit the activation of microglia and secretion of pro-inflammatory mediators. We postulate that part of this inhibition process may be mediated through the JNK pathway and lead to neuroprotection. All fluorescent microphotographs of double labelling are from the CA1 hippocampal region. Scale bar: 100 µm.

Altogether, these observations suggested that the activation of JNK leading to c-Jun phosphorylation is not predominant in microglia, and that treatment with D-JNKI1 after OGD could modify microglial activation and localization within the CA1 neuronal layer.

As we observed activation of p-c-Jun in neurons and not in microglia, and that D-JNKI1 was mainly localized in neurons but also in some microglial cells, we may assume that D-JNKI1 is more likely to modulate microglial activation *in vitro* indirectly.

Intriguingly, the *in vivo* results suggest that treatment with D-JNKI1 following ischemia does not reduce the recruitment and activation of microglia in the ischemic brain territory at 48 h. However, D-JNKI1 appears to alter microglial activation and localization following OGD *in vitro* (Figure 16E: schematic presentation). While this may partly be due to the *in vitro* hypoxia model not reproducing the *in vivo* ischemia model, these observations need to be further explored, for instance by quantitative measurements of inflammatory cytokines released by microglia, in animals submitted to experimental ischemia with or without treatment. This issue will be addressed in the second part.

(These results have been in part published as: JNK inhibition and inflammation after cerebral ischemia. Benakis C, Bonny C, Hirt L. *Brain Behavior and Immunity* 24 : 800–811, 2010).

SECOND PART: EFFECT OF NEUROPROTECTION ON THE SECRETION OF INFLAMMATORY MEDIATORS

We showed previously that accumulation and activation of microglia within the ischemic tissue is not influenced by D-JNKI1. Besides, our *in vitro* results suggested that the modification of microglial morphology we observed after treatment could probably be due to subtle local changes, such as the release of pro- and anti-inflammatory mediators (Benakis et al., 2010). Regarding these findings we hypothesized that the neuroprotective action of D-JNKI1 could be due partly to the modulation of inflammatory mediators release (Figure 17). Furthermore, as we could not detect the peptide by immunohistochemistry in the brain tissue after i.v injection of 0.1 mg/kg, we addressed the question of a potential effect of D-JNKI1 on the peripheral immune system. Therefore we were interested to investigate the release of inflammatory mediators after cerebral ischemia and the effect of the neuroprotective peptide D-JNKI1 on this release.

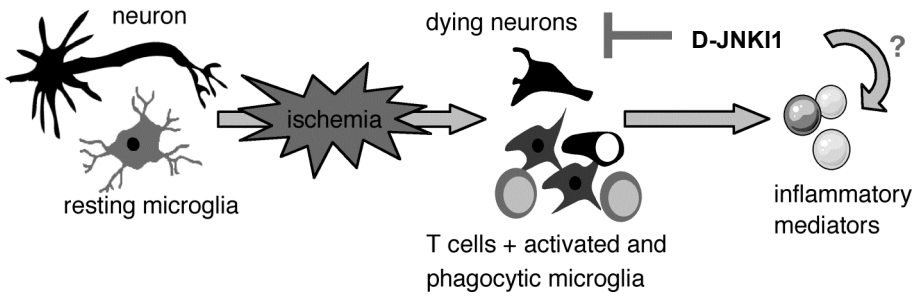


Figure 17. Schematic presentation of the hypothetic role of D-JNKI1 after an ischemic insult

After ischemia neurons are starting to degenerate and inflammatory cells such as microglia become activated and change their morphology to a less ramified and phagocytic state. Leukocytes such as T lymphocytes are also recruited in the ischemic tissue. D-JNKI1 inhibits neuronal death but not microglial activation. We asked whether D-JNKI1 influences the release of inflammatory mediators such as cytokines and chemokines. We suggest that D-JNKI1 could protect neurons from apoptosis by modulating the secretion of inflammatory mediators.

Regarding conflicting data on the detrimental or beneficial role of the cytokine IL6 and the chemokine KC in cerebral ischemia (see Introduction sections 1.5.3 and 1.5.4) and based on recent findings published by others (Chapman et al., 2009) we chose to analyze the secretion of these two inflammatory molecules in the systemic circulation, in the brain and other peripheral organs (spleen, lung and liver). We performed an ELISA at several time points in sham (vehicle or D-JNKI1) and MCAO (vehicle or D-JNKI1) mice. A schematic diagram of the experimental procedure is shown in Figure 18.

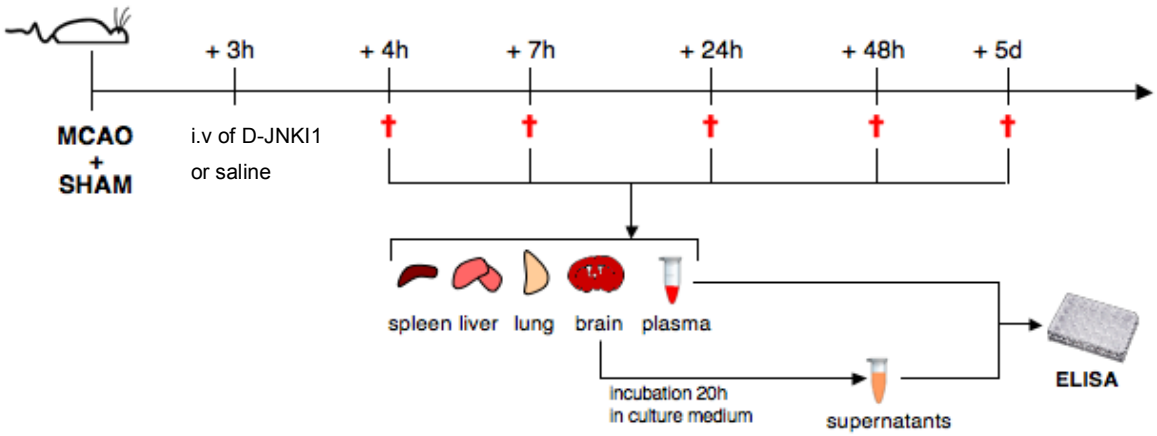


Figure 18. Schematic diagram of the analysis of the release of inflammatory mediators
Sham or MCAO mice were treated with saline solution or 0.1mg/kg of D-JNKI1 3 h after the start of the surgical procedure. Mice were sacrificed at several time points. Plasma, brain and peripheral organs were isolated. Plasma samples and supernatants of organ slices incubated for 20 h in a culture medium were assessed by ELISA.

3.7 TRANSIENT MCAO INDUCES A RAPID SYSTEMIC SECRETION OF INFLAMMATORY MEDIATORS

The secretion of inflammatory mediators after MCAO has never been investigated in outbred CD1 mice. Therefore, we assessed the time course of IL6 and KC release in sham and MCAO mice, in order to identify the relevant time points for the analysis of D-JNKI1 treatment on cytokine/chemokine release. First, we performed transient MCAO for thirty minutes and sacrificed the mice after 4 h (n = 6), 7 h (n = 10), 24 h (n = 6), 48h (n = 9) and 5 d (n = 6) of reperfusion. Sham mice (n = 5 to 8, except at 24 h, n

= 3) were anesthetized for the same duration as ischemic mice and were sacrificed at the same time points. (Table 2 in the Appendix shows measurements of cerebral blood flow, weight, and temperature of sham and MCAO mice. Weight, temperature and neuroscore at sacrifice are also listed). Control mice (no surgical intervention) were used as an indicator of the basal level (n = 7). Plasma samples were collected and assessed by ELISA for the cytokine IL6 and the chemokine KC. We found a marked plasma release of both inflammatory mediators very early after ischemia onset (Figure 19). Already four hours after the injury, the level of IL6 in the systemic circulation was significantly higher than in sham mice ($p < 0.05$). The increase was still significant at 7 h ($p < 0.05$) (Figure 19A). From 24 h to 48 h IL6 release decreased to sham plasma levels. As late as five days after ischemia, secretion of IL6 in the plasma increased again to an almost significant level compared to sham mice (sham versus MCAO: $p = 0.054$). Figure 19B showed that the KC plasma release followed closely the secretion pattern of IL6, with a very early induction in the plasma 4 h ($p < 0.05$) and 7 h ($p < 0.01$) after MCAO which dramatically decreased between 24 h and 48 h. Five days after the occlusion we found that KC was once again significantly secreted in the plasma of MCAO mice compared to sham mice ($p < 0.05$).

In accordance with previous studies, these results indicate a significant plasma release of IL6 and KC early after cerebral ischemia compared to sham mice (Chapman et al., 2009; Offner et al., 2006).

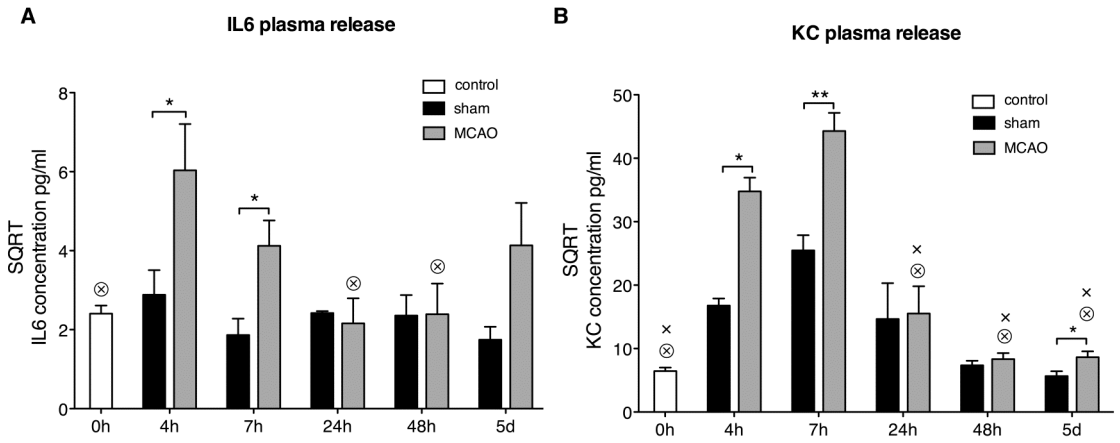


Figure 19. Transient MCAO induces a rapid release of IL6 and KC in the plasma

The graphs show the square root of IL6 and KC concentrations in pg/ml at different time points. (A) Plasma IL6 was induced 4 and 7 hours after MCAO at a significantly higher level than sham mice. At 24 h and 48 h IL6 concentration of stroke mice returns to basal level and tends to increase 5 days after the occlusion (sham versus MCAO: $p = 0.054$). (B) Plasma KC was also induced early after MCAO compared to sham mice and decreased from 24 h to 5 days. At this time point KC concentration was significantly higher than sham mice (sham versus MCAO: $p = 0.03$). Control mice: *n = 7*, sham mice: *n = 3 to 8*, MCAO mice: *n = 6 to 10*. *$p < 0.05$, **$p < 0.01$ indicate a significant difference between MCAO mice and sham mice. Results are represented as mean ± SEM. ⊗ and × denote a significant difference from the peak concentration at 4 h and 7 h in MCAO mice, respectively

In Figure 23 we described changes of IL6 and KC release as a function of time. It is clearly shown that in MCAO mice plasma release of IL6 at 4 h is significantly higher than at 24 h ($p < 0.05$) and 48 h ($p < 0.05$) (Figure 23A2) and that this significant difference is lost at 5 d. We observed a similar time course for KC plasma release after MCAO (Figure 23B2). The secretion of KC at 4 h and 7 h is significantly higher than at all late time points (24 h, 48 h and 5 d) even though there is a significant increase of KC at 5 d compared to sham mice as described above (see Figure 19B). Furthermore, we found a significant release at 4 h, 7 h and 24 h in sham group compared to a control group (0 h, no surgical intervention, Figure 23B1) as well as at 4 h and 7 h in MCAO mice. These findings suggest that KC is transiently secreted early in the plasma and that this might be due at least in part to the stress caused by the surgical procedure.

3.8 BRAIN RELEASE OF INFLAMMATORY MEDIATORS FOLLOWS SYSTEMIC INFLAMMATION

In order to investigate the *de novo* secretion of cytokines and chemokines in the brain induced by the ischemic insult, we analyzed by ELISA the supernatants of brain slices from sham and MCAO mice incubated for 20 h in a culture medium. We found a significant elevation of IL6 (Figure 20A) but not KC (Figure 20B) in the brain of MCAO mice compared to sham mice at late time points. Indeed, at 24 h after ischemia, IL6 tended to increase compared to sham mice ($p = 0.06$) and was significantly

different at 48 h ($p < 0.05$). A slight increase was also observed at 5 d, although this did not reach statistical significance ($p = 0.12$). Brain KC release had a slight though non-significant tendency to increase at 24 h ($p = 0.19$) and 48 h ($p = 0.08$) after MCAO. Considering the temporal evolution of cerebral cytokine and chemokine secretion, in Figure 23C2 and 23D2 we showed that the brain release of IL6 and KC increased significantly at 7 h compared to 0 h (control mice) and 4 h in MCAO mice, and remained elevated at late time points, especially for KC (KC at 4 h versus 48 h : $p < 0.05$, KC at 4 h versus 5 d : $p < 0.05$, IL6 at 4 h versus 48 h : $p = 0.056$). In sham mice, in contrast, cerebral release of IL6 and KC did not differ from control mice and any time point.

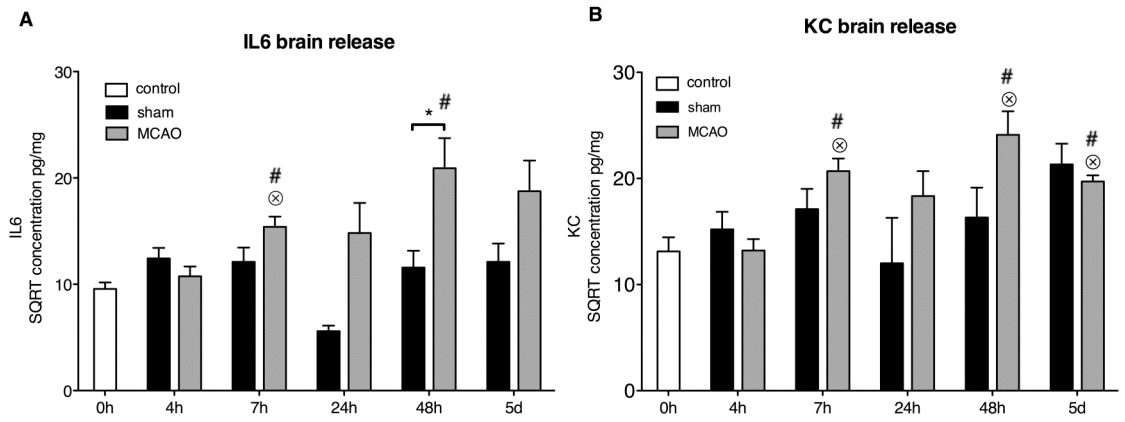


Figure 20. Transient MCAO induces a late release of IL6 but not KC from the brain

Concentrations of IL6 and KC were measured in the supernatants of brain slice cultures and normalized to total protein concentration. The graphs show the square root of IL6 and KC concentrations in pg/mg at different time points (A) Secretion of IL6 from the brain tended to increase at 24 h (sham versus MCAO: $p = 0.06$) with a significant difference between sham and MCAO mice 48 h after the occlusion. (B) Brain KC was not significantly different from sham mice at all time points. Control mice: *n = 7*, sham mice: *n = 3 to 8*, MCAO mice: *n = 6 to 10*. *$p < 0.05$ indicates a significant difference in MCAO mice and sham mice. Results are represented as mean ± SEM. # and ⊗ denote a significant difference from the peak concentration at 0 h and 4 h in MCAO mice, respectively.

Besides, we observed that the time course of brain secretion of IL6 correlates with the evolution of the apparent lesion size as assessed by TTC staining (Figure 21A). Indeed,

TTC staining of brain slice after MCAO showed a clearly outlined lesion at 24 h and 48 h which corresponds to higher brain release of IL6. Besides the plasma release of IL6 and KC does not correlate with the outlined lesion size. At early (4 h and 7 h) and late (5 d) time points the lesion was not detectable by TTC staining. These time points correspond to higher plasma release of IL6 and KC, as schematically illustrated in Figure 21B (bottom).

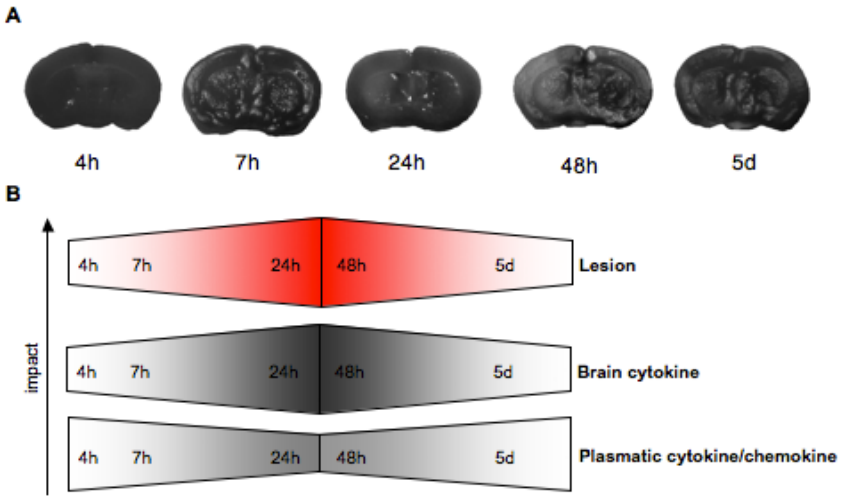


Figure 21. Lesion size as a function of time after MCAO

(A) Lesion size is evaluated qualitatively using TTC staining. Mice are sacrificed at 4 h, 7 h, 24 h, 48 h and 5 d. Brains are removed, sliced in 2-mm-thick sections and one anterior slice is stained with 2% TTC. Representative brain slices for one animal at each time point are shown. Lesion appears in white at 24 h and 48 h in the left hemisphere. No distinguishable lesion is seen at all other time points. (B) Simplified diagram showing the evolution of the lesion and cytokine release in the plasma and in the brain.

Preliminary analysis of IL6 and KC release in the supernatants of spleen, lung and liver suggest a time course similar observed to that of the plasma in the spleen and liver, and no clear direction of change in the lung (data not shown). Further investigations are required to confirm these impressions.

Altogether our data showed that systemic inflammation after cerebral ischemia occurred before brain inflammation, and that the latter correlates with the apparent evolution of the lesion size. Furthermore, we found that *de novo* brain release of IL6

occurs at 48 h when the lesion size is clearly outlined, and that the time course of its cerebral secretion seems more protracted than in the periphery.

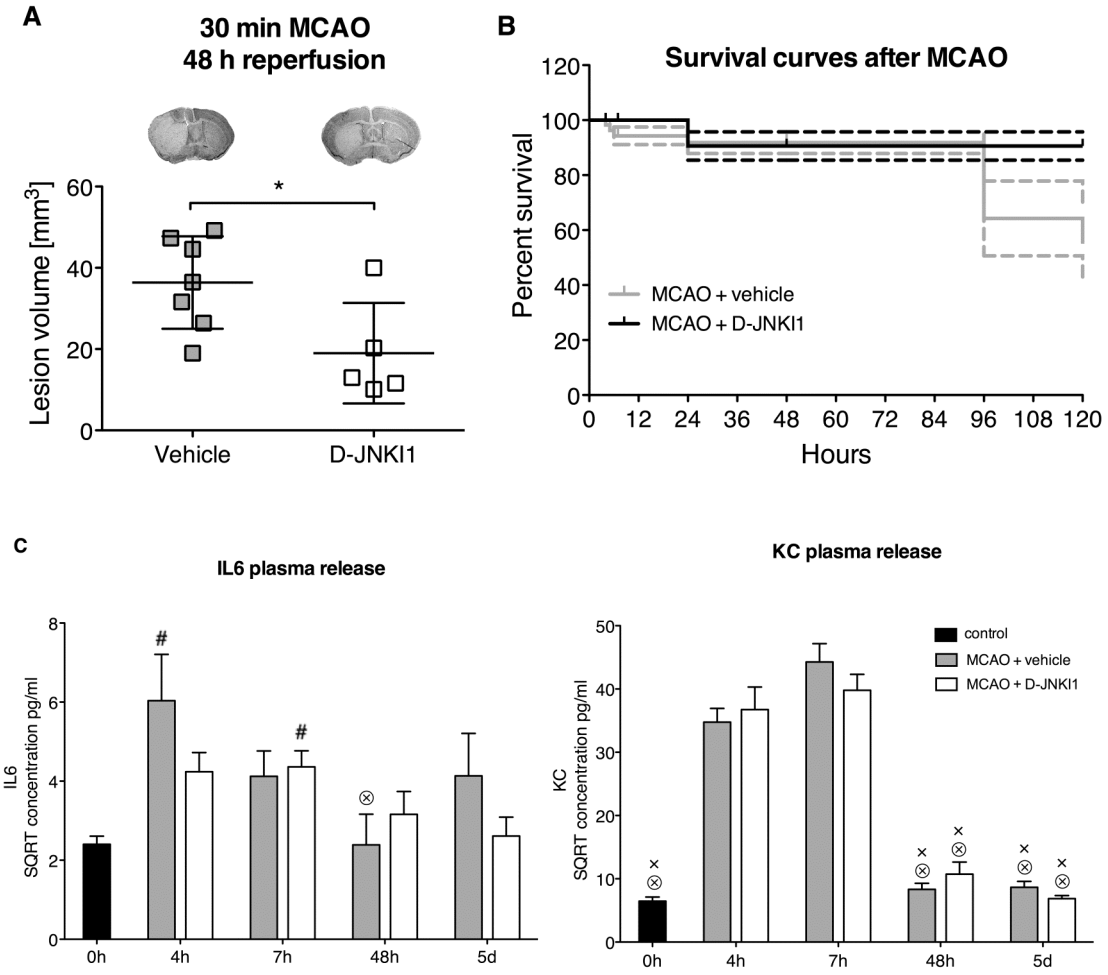
3.9 NEUROPROTECTION MEDIATED BY D-JNKI1 48 H AFTER INJURY IS INDEPENDENT OF PLASMA AND BRAIN CYTOKINE RELEASE

To analyze whether D-JNKI1 could modulate the release of inflammatory mediators after cerebral ischemia, we quantified IL6 and KC release in the plasma and brain supernatants of mice treated with D-JNKI1. First, in a separate set of experiments mice were subjected to 30 min MCAO and injected i.v. with 0.1 mg/kg of D-JNKI1 or saline solution 3 h after MCAO onset. Forty-eight hours after surgery, the lesion volume was quantified on serial coronal brain slices. The direct lesion volume was 36.4 ± 4.3 mm^3 in saline-injected mice (n = 7), and 19.0 ± 5.5 mm^3 in D-JNKI1-treated mice (n = 5), providing a significant 50% reduction of the lesion size (p = 0.03), corroborating our previous findings (Benakis et al., 2010); (Figure 22A). (Table 3 in the Appendix shows measurements of cerebral blood flow, weight, and temperature of mice included in this study. Weight, temperature and neuroscore at 48 h are also listed).

Plasma samples of mice subjected to 30 min MCAO and treated with D-JNKI1 were then isolated at 4 h (n = 7), 7 h (n = 7), 48 h (n = 8) and 5 d (n= 7) after ischemia and tested by ELISA for IL6 and KC (see Figure 18 for experimental procedure). At each time point, we did not find any significant difference between MCAO+D-JNKI1 and MCAO+vehicle for either of these inflammatory mediators (Figure 22C). Four hours after the injury, D-JNKI1 induced a non-significant tendency towards a reduction in IL6 release (MCAO+vehicle versus MCAO+D-JNKI1: p = 0.26), but not KC. Five days after the injury, we observed again a slight, non-significant reduction of IL6 plasma release with D-JNKI1 treatment (MCAO+vehicle versus MCAO+D-JNKI1: p = 0.27). We then assessed the release of IL6 and KC in the supernatants of brain samples of mice treated with D-JNKI1. We chose to analyze IL6 and KC release at three different time points (7 h, 48 h and 5 d) as we hypothesized that they would be the more interesting time points based on our previous results after MCAO (Figure 20). We did not find a significant difference of IL6 nor KC brain release at any time point as

shown in Figure 22D. However, a slight non-significant reduction of IL6 brain release was seen at 5 d (MCAO+vehicle versus MCAO+D-JNKI1: p = 0.25). These results suggest that D-JNKI1 does not significantly modulate the secretion of IL6 and KC after an ischemic insult, neither systemically nor in the brain.

To rule out an effect of the peptide itself on the release of inflammatory mediators, we tested the secretion of IL6 and KC in sham mice treated with D-JNKI1. Plasma and brain supernatant samples of sham mice injected with D-JNKI1, sacrificed at 48 h, were evaluated for IL6 and KC. We did not find any significant release of both inflammatory mediators between sham+vehicle and sham+D-JNKI1 48 h after the surgical procedure (data not shown).



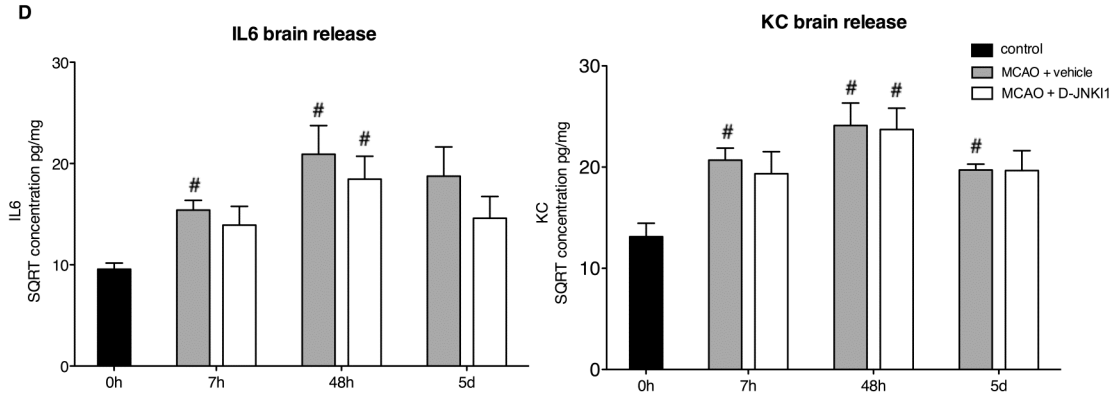
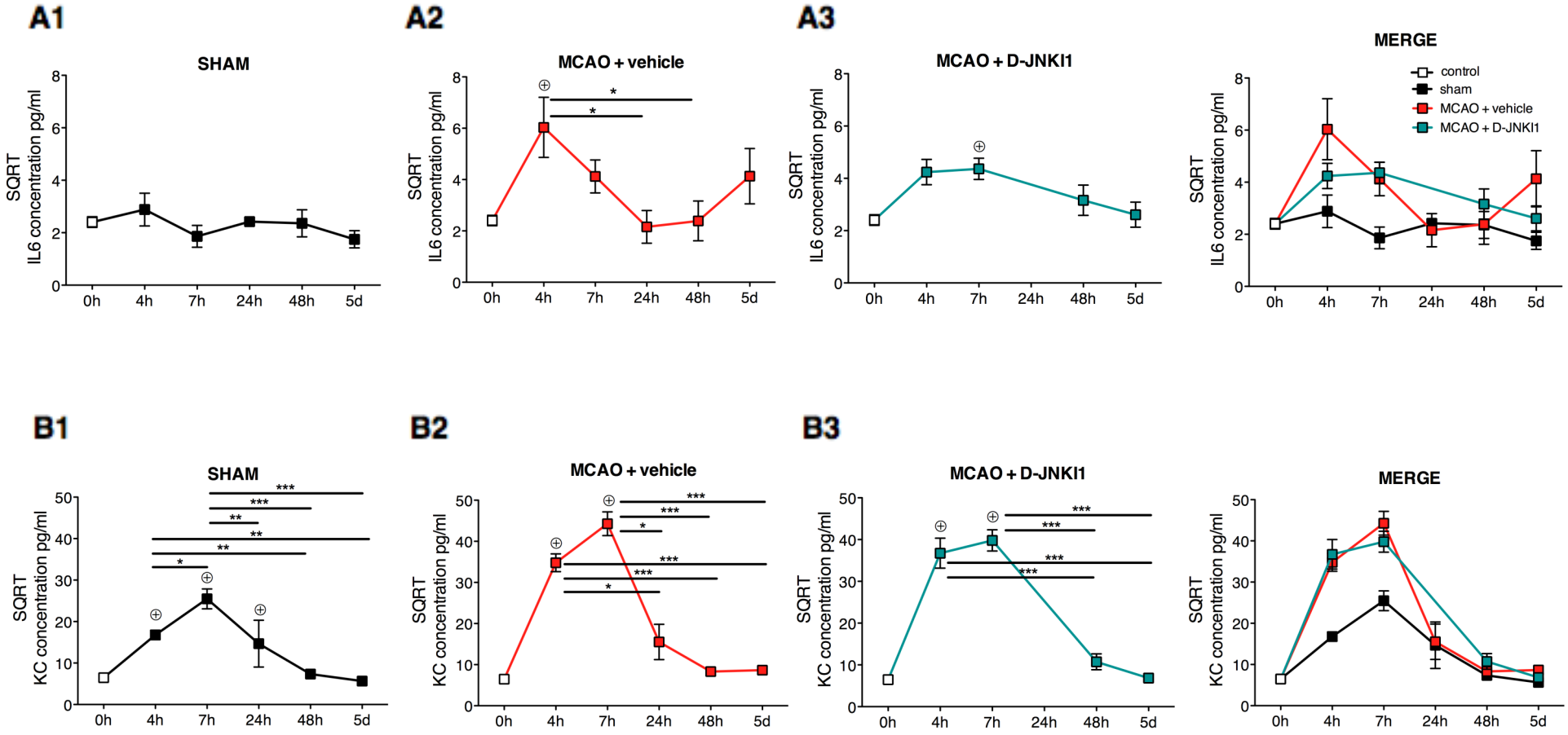


Figure 22. Effect of D-JNKI1 on lesion volume, survival and cytokine/chemokine secretion

(A) Late intra-venous administration of 0.1 mg/kg D-JNKI1 after transient occlusion of the MCA leads to neuroprotection. MCAO was induced for 30 minutes and the lesion volume was measured on consecutive coronal brain slices 48 h after reperfusion. A significant reduction of the lesion size was observed in treated mice compared to vehicle mice ($p < 0.05$). Representative examples of Nissl staining are shown above. (B) Survival curves show no significant difference between mice treated with D-JNKI1 or vehicle from the end of surgery until 96 hours. At 5 days post-surgery mice not treated with D-JNKI1 have a worse outcome compared to treated mice. (C) IL6 and KC release were measured at 4 h, 7 h, 48 h and 5 days in plasma samples of mice subjected to MCAO and treated with D-JNKI1. No statistical difference was found between MCAO+vehicle and MCAO+D-JNKI1. (D) IL6 and KC release were measured at 7 h, 48 h and 5 d in brain slice supernatants of mice treated with D-JNKI1. No statistical difference was found between treated and vehicle animals. Control mice: *n = 7*, MCAO+vehicle mice: *n = 6 to 10*, MCAO+D-JNKI1 mice: *n = 7 to 8*. Results are represented as mean ± SEM. #, × and ⊗ denote a significant difference from the peak concentration at 0 h, 4 h and 7 h, respectively.

In order to complement the demonstration of a reduction in lesion size by D-JNKI1 treatment, we analyzed the survival of MCAO+vehicle compared to MCAO+D-JNKI1 mice using Kaplan-Meier analysis (Figure 22B). A total of 102 animals were included in this analysis (MCAO+vehicle: $n = 57$, MCAO+D-JNKI1: $n = 45$). The survival appears better in the D-JNKI1-treated group, the overall difference was not statistically significant (Log-rank Mantel-Cox Test: $p = 0.19$; Table 4 in the Appendix shows survival proportions in each group, no death was reported in the sham group).

Interestingly, our results showed that neuroprotection mediated by D-JNKI1 measured 48 h after the injury is independent of early systemic inflammation as well as late brain inflammation. Looking carefully at the survival curves of MCAO+D-JNKI1 mice, no death was noted from 24 h in this group compared to the MCAO+vehicle group. In addition, we observed a slight decrease of IL6 plasma and brain release as a function of time in the MCAO+D-JNKI1 group, especially at 5 d after reperfusion. Thus, we could formulate the hypothesis that a sustained or a secondary inflammatory response would be unfavorable for survival prognosis at later time points. This hypothesis needs to be further explored, as for instance by the investigation of IL6 and KC release at 7 days and 14 days after ischemia.


A1
SHAM
SQRT IL6 concentration pg/ml
0h 4h 7h 24h 48h 5d
A2
MCAO + vehicle
A3
MCAO + D-JNKI1
MERGE
control
sham
MCAO + vehicle
MCAO + D-JNKI1
B1
SHAM
SQRT KC concentration pg/ml
B2
MCAO + vehicle
B3
MCAO + D-JNKI1
MERGE

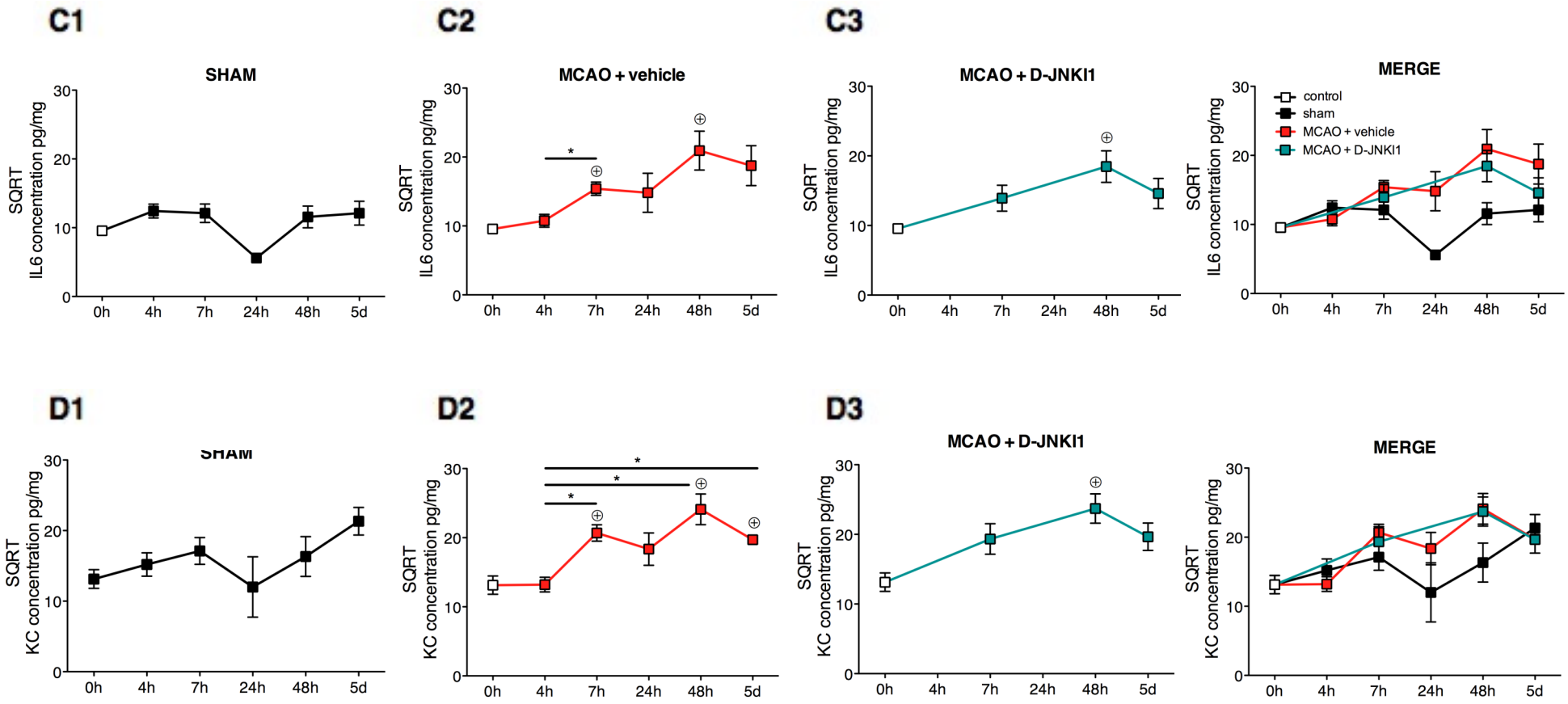

C1
SHAM
C2
MCAO + vehicle
C3
MCAO + D-JNKI1
MERGE
control
sham
MCAO + vehicle
MCAO + D-JNKI1
SQRT IL6 concentration pg/mg
D1
SHAM
D2
D3
MCAO + D-JNKI1
MERGE
SQRT KC concentration pg/mg
0h
4h
7h
24h
48h
5d

Figure 23. Effect of time on IL6 and KC release

Secretion of IL6 and KC in the plasma and from the brain in sham mice and mice subjected to MCAO treated with D-JNKI1 or saline solution were first compared to control mice. ◦P<0.05 indicates a significant difference in sham, MCAO+vehicle and MCAO+D-JNKI1 mice versus control mice (0 h). For each group, cytokine/chemokine release was analysed as a function of time. *p < 0.05; **p < 0.01; ***p < 0.001 indicate a significant difference between time points. (A) In the plasma of MCAO mice treated with D-JNKI1 (A3) or vehicle mice (A2) there is a significant release of IL6 at early time points compared to control mice (A2: 4 h, p = 0.037; A3: 7 h, p = 0.046). In MCAO mice, the secretion of IL6 at 4 h is significantly higher than at 24 h and 48 h (A2). There was no difference between different time-points in treated mice (A3) as well as in sham mice (A1). (B) The KC release in the plasma of sham (B1), MCAO+vehicle (B2), MCAO+D-JNKI1 (B3) was significantly different than in control mice at early time-points (B1: 4 h, p = 0.002; 7 h, p < 0.0001; 24 h, p = 0.036; B2: 4 h, p < 0.0001; 7 h, p < 0.0001; B3: 4 h, p = 0.001, 7 h, p < 0.0001). In all three groups the release of KC is significantly higher at early time points (4 h and 7 h) than at late time points (24 h or 48 h and 5 d). (C and D) The IL6 and KC release from brain tissue is significantly higher at 48 h after MCAO+vehicle (C2, D2) and MCAO+D-JNKI1 (C3, D3) compared to control mice (0 h). (C2) In MCAO mice, the secretion of IL6 at 7 h is already significantly higher than at 4 h and tends to increase at 48 h (4 h versus 48 h: p = 0.056). There was no difference between different time-points in treated mice (C3) or in sham mice (C1). (D2) In MCAO mice, the secretion of KC at 7 h, 48 h and 5 d is significantly higher than at 4 h. No difference between different time-points was found in treated mice (D3) or in sham mice (D1). Control mice: *n = 7*; Sham mice: *n = 3 to 8*; MCAO+vehicle mice: *n = 6 to 10*; MCAO+D-JNKI1 mice: *n = 7 to 8*. Results are represented as mean ± SEM.

4. DISCUSSION

In the first part of this study, we showed that microglia was activated throughout the lesion 48 h after experimental stroke. However, in the context of neuroprotection, neither was there an inhibition of the activation of microglia nor a reduced number of activated microglia 48 h after the inhibition of neuronal cell death. Thus we showed that protection of neurons is independent of the activation of microglia in the ischemic tissue. In the second part of this project, we emphasized that early systemic release of inflammatory mediators is not a marker for irreversible brain lesion after experimental stroke. Indeed we obtained neuroprotection measured at 48 h even though there was an increase of IL6 and KC plasma levels very early after the onset of cerebral ischemia. Furthermore we found that the IL6 concentration in the brain was not different in groups given intravenous saline solution or the neuroprotective peptide D-JNKI1, whereas we obtained a significant reduction of the lesion size in the treated group. Altogether, we showed that the inhibition of the JNK signaling pathway using D-JNKI1 does not lead: 1) to an inhibition of microglial activation in terms of morphology and cell number, 2) to a decreased level of early systemic release of cytokines/chemokines, 3) to a reduction of *de novo* brain secretion of inflammatory mediators, despite a reduction of the lesion volume. This strongly suggests that brain inflammation (showed particularly by the activation of microglia and the release of IL6) is not linked to the severity of the lesion, and that early systemic inflammation is independent of the neurological outcome.

FIRST PART: EFFECT OF NEUROPROTECTION ON INFLAMMATORY CELLS

In the normal healthy brain, microglial cells are known to continually screen their microenvironment by quickly moving their processes (Kreutzberg, 1996; Nimmerjahn et al., 2005; Hanisch & Kettenmann, 2007). Controlled activation of microglia induces the production of trophic factors and elimination of cell debris, leading to the protection of surrounding cells (Raivich et al., 1999; Streit et al., 1999; Aloisi, 2001). An ischemic

insult could cause an exaggerated activation of microglia with excessive release of inflammatory mediators and uncontrolled phagocytotic function (Kreutzberg, 1996; Rossum & Hanisch, 2004). It was postulated that this over-activation might be deleterious for the survival of neurons whereas controlled activation of microglia might be neuroprotective (Biber et al., 2007). The lack of knowledge on the role of microglial activation after brain ischemic injury led us to investigate how microglia react in a neuroprotective condition and if they participate in brain injury or repair.

Based on the hypothesis that inflammatory cells are deleterious for tissue recovery, we raised the question whether D-JNKI1 protects neurons through the inhibition of microglial activation. In this first part, we investigated in experimental models of cerebral ischemia whether the neuroprotective effect of D-JNKI1 was due to an inhibition of microglial activation and second whether it was dependent of the JNK signaling pathway.

4.1 EFFECT OF NEUROPROTECTION ON MICROGLIAL ACTIVATION

Our results indicate that 0.1 mg/kg of D-JNKI1 was strongly neuroprotective when injected i.v. 3 h after transient MCAO. Despite efficiently attenuating neuronal death by inhibition of the JNK pathway, D-JNKI1 did not reduce the accumulation of microglia *in vivo* as shown by the quantification of CD11b-positive microglial cells (Figure 13C). These interesting findings indicate that neuroprotection due to D-JNKI1 treatment in this transient ischemic model is independent of the accumulation of microglia observed 48 h after injury. Furthermore, our analysis of correlation between the infarct volume and the number of microglia in the ischemic tissue of vehicle animals reveals that there is a strong correlation: the larger the lesion, the more activated microglial cells are present in the infarct. Linear regression comparison between groups showed no statistical difference. However, in the group treated with D-JNKI1, the correlation between the lesion volume and number of microglia was not significant, in contrast to the vehicle group (Figure 13D). Overall, while we cannot unequivocally state that microglia are neuroprotective, we suggest that their presence in the ischemic region might not be detrimental. Indeed, others showed a significant

improvement of behavioral tasks by intra-cerebroventricular injection of labeled exogenous microglia 1 h after induction of MCAO in rats, compared to non-injected rats (Kitamura et al., 2005). Other authors using transgenic mice with a selective ablation of endogenous microglia found an exacerbated size of the ischemic lesion after transient MCAO compared with wild type mice (Lalancette-Hébert et al., 2007). Both studies used drastic interventions which might explain the measured effect: an obvious neuroprotective effect is seen in models when microglia are injected in the brain parenchyma after cerebral ischemia, and an increased damage is observed when microglia are depleted.

To complement the investigation of the effect of D-JNKI1 on microglial accumulation following cerebral ischemia, we performed a qualitative study aimed at detecting morphological changes of microglia in vehicle and treated animals subjected to MCAO. We assumed that neuroprotection mediated by D-JNKI1 might inhibit the change of microglial morphology from a resting state towards an activated or ameboid state, the latter commonly associated with neurotoxic activity. Our results indicate no morphological difference between vehicle and D-JNKI1 animals subjected to ischemia (Figure 13A). Microglia exist in three distinct morphologies correlating with their state of activation, as schematically illustrated in Figure 4. In the context of a tissue injury, it is expected that a graded microglial response will be observed, associated with several morphologies instead of one single state. Indeed microglia react rapidly to subtle local changes caused by damaged neurons. Therefore, one might not be able to see an overall morphological change within the total ischemic infarct, since the degree of microglial activation could vary with the severity of the injury (Raivich et al., 1999; Schwartz et al., 2006; Lynch, 2009). Indeed, it is suggested that the population of ameboid microglia is more abundant in the irreversibly damaged tissue (necrotic cell death) whereas activated ramified microglia might be recruited into the salvageable peri-infarct region (apoptotic cell death) (Zhang et al., 1997; Stoll et al., 1998). Therefore, it would be useful to discriminate between these different regions and quantify the local effect of D-JNKI1 on the morphologic changes of microglia by using different markers for each state of activation. Indeed, previous work showed a suppression of microglial

activation and proliferation in a delimited area: the cortex boundary zone, after MCAO using roscovitine as a cell cycle inhibitor leading to neuroprotection (Zhang et al., 2009). Furthermore, it is still unclear whether the different 'grades' of microglial response to brain tissue injury are associated with a specific functional role. Thus, one type of morphology does not necessarily correspond to one specific phenotype. Even though D-JNKI1 does not inhibit the activation of microglia based on a qualitative morphological study, we cannot rule out a possible effect of the neuroprotective peptide on the functional modification of microglia, as for instance a change from a pro-inflammatory towards an anti-inflammatory secretion profile.

Altogether, D-JNKI1 inhibits neuronal death without affecting the accumulation of microglia, and without an obvious effect on microglial activation state, suggesting that the activation of microglia does not necessarily exacerbate neuronal damage induced by transient cerebral ischemia.

4.2 EFFECT OF D-JNKI1 ON T LYMPHOCYTES AND ASTROCYTES

In accordance with previous reports, we found accumulation of T lymphocytes (TCRβ+ cells) and dendritic cells (CD11c+) in the ischemic tissue 48 h after MCAO (Kato et al., 1996; Raivich et al., 1999; Kostulas et al., 2002; Reichmann et al., 2002; Gelderblom et al., 2009). The release of inflammatory mediators from activated glial cells and the opening of the blood brain barrier (BBB) following stroke (Barone & Feuerstein, 1999) induced the recruitment of circulating leukocytes (granulocytes, monocytes/macrophages, lymphocytes). Several publications have described that blocking leukocyte infiltration decreases secondary injury linked to inflammatory process by inhibiting the formation of reactive oxygen species (nitric oxide, superoxide) and preventing the breakdown of the blood brain barrier which leads to hemorrhagic complications (Iadecola et al., 1995; Parmentier et al., 1999; Barone & Feuerstein, 1999; Zoppo & Hallenbeck, 2000; Zoppo et al., 2000; Rosell et al., 2008). Thereby, the reduction of T lymphocytes in the ischemic tissue might be neuroprotective. Activation of the JNK pathway leads to the transcription of inflammatory molecules, such as chemokines which could further drive the activation

and recruitment of T lymphocytes. The inhibition of the JNK pathway in microglia reduces in some cases the expression of inflammatory mediators (see Introduction, section 1.7.2). Therefore, we could hypothesize that the reduction of inflammatory mediators through the inhibition of JNK would result in a diminution of T lymphocyte recruitment in the ischemic parenchyma. Thus, D-JNKI1 would indirectly modify the T cell response after ischemia, and therefore decrease the neuronal damage. The astrocytic response after stroke is not well understood yet, since astrocytes secrete pro-apoptotic signals, inflammatory mediators as well as growth factors (Anderson et al., 2003). It has been shown that astrocyte proliferation after endothelin-1 stimulation depends on c-Jun induction (Gadea et al., 2008). In agreement with these findings we showed an activation of the nuclear target of JNK, phospho-c-Jun, in reactive astrocytes after MCAO. D-JNKI1 could therefore modulate the JNK pathway in astrocytes and probably the inflammatory response after stroke.

We and others clearly demonstrated that microglia and other inflammatory cells were activated in the ischemic tissue. All these cells could potentially be influenced by the inhibition of the JNK pathway (Waetzig et al., 2005; Raivich & Behrens, 2006). Therefore it would be of great interest to investigate the possible effect of D-JNKI1 on T lymphocytes, DCs and astrocytes in our model of brain ischemia. Indeed, based on the hypothesis that modulation of inflammation could be neuroprotective, it would be interesting to investigate the effect of D-JNKI1 on different cell types rather than focusing on microglial cells only.

4.3 ACTIVATION OF THE JNK SIGNALING PATHWAY IN MICROGLIA

As microglia are capable of various responses depending on the type of injury, and since changes in morphology and upregulation of surface expression markers do not always reflect microglial activation, it will be necessary to go on to investigate its function as for instance the release of inflammatory mediators (Feuerstein et al., 1998; Hanisch, 2002; Garden & Möller, 2006; Clausen et al., 2008). Previous studies have reported that the ischemic damage is attenuated by inhibiting pro-inflammatory cytokines or transcription factor such as nuclear factor-κB (NF-κB), by diminishing

free radicals generated by inducible nitric oxide synthase (iNOS) or cyclooxygenase- 2 (COX-2), (Iadecola et al., 1995; Yang et al., 1998; Parmentier et al., 1999; Nijboer et al., 2009). As the JNK pathway is also involved in the regulation of inflammatory molecules (Hidding et al., 2002; Waetzig et al., 2005), D-JNKI1 could therefore modulate cytokine secretion. Several studies have described the involvement of the mitogen-activated protein kinase pathway (MAPKs family, including JNK, ERK and p38) in the regulation of inflammatory mediators [for review see (Kaminska, 2005) and Introduction, section 1.7]. *In vitro,* the JNK pathway is activated in microglial cultures stimulated by TNFα, thrombin or LPS. CEP-11004 (an inhibitor of mixed lineage kinases) reduces LPS-induced phosphorylation of JNK and c-Jun and attenuates microglial morphological changes (Hidding et al., 2002) and SP600125 (an ATP-competitive JNK inhibitor) reduced microglial metabolic activity and proliferation (Waetzig et al., 2005). Here, we report for the first time that phospho-c-Jun was activated in microglia after transient MCAO in mice, which led us to test whether D-JNKI1 could modulate microglial activity through the regulation of the JNK pathway. Unless we found activation of the JNK pathway in microglia, D-JNKI1 could not modify its activation state. Thus JNK may not be the predominant or only pathway required for microglial activation in the brain after cerebral ischemia. As JNK and p38 are up-regulated after stress, one could imagine that inhibition of more than one pathway would be necessary for complete inhibition of microglial activation. An inhibitor of p38 mitogen-activated protein kinase (SB239063) has been shown to reduce microglial activation after OGD in hippocampal cultures when administrated 2 h before and until 24 h after the deprivation (Strassburger et al., 2008). Thereby, combined inhibitors of the MAPK pathways could affect more significantly the activation of microglia *in vivo*.

4.4 EFFECT OF D-JNKI1 ON MICROGLIA *IN VITRO*

With the aim to understand the molecular mechanisms of the JNK pathway in microglia, we investigated the role of D-JNKI1 *in vitro*. In our OGD model of ischemia, we showed that the peptide prevented neuronal cell death in the CA1, reflected by a reduction of propidium iodide uptake, as previously described (Hirt et al., 2004). We found a strong activation of phospho-c-Jun in CA1 neurons 48 h after OGD. Treatment with D-JNKI1 did not completely block c-Jun activation but rescued neurons from OGD-induced death. It is well described that levels of activated JNK are found in neurons under basal conditions, indicating that the JNK pathway is also important for other metabolic processes (Harris et al., 2002; Besirli et al., 2005), suggesting that a complete suppression of c-Jun could impair different important functions and not be neuroprotective. In agreement with our results, experimental studies on mice harboring conditionally inactivated c-Jun and non-phosphorylating c-Jun mutant do not show a reduction of the lesion size after transient MCAO (Vogel et al., 2007).

In slices subjected to OGD and treated with D-JNKI1, we found a co-localization of D-JNKI1 with neuronal markers and, to a much lesser extent, with microglia. Interestingly, we observed morphological changes of microglia *in vitro*: while microglial cells were in a well ramified state in control slices, they became more rounded after OGD, and D-JNKI1 inhibited this morphological change. The JNK pathway modulates the phosphorylation of actin and microtubules and could thereby modify the microglial morphology (Waetzig et al., 2005). Besides modification of microglial morphology after D-JNKI1 treatment *in vitro*, we found a change in microglial localization after OGD and after OGD+D-JNKI1. In control slices microglia were homogenously distributed whereas after OGD microglia were predominantly localized within the damaged neuronal CA1 layer. Treatment with D-JNKI1 modified slightly the localization of microglia, which were found at the boundary of the neuronal CA1 layer as well. Modification of microglial cell localization at the site of injury has been previously reported in hippocampal slice cultures (Heppner et al., 1998; Montero

Domínguez et al., 2009). Altogether, OGD induced changes in microglial morphology (ramified to ameboid) and accumulation in the degenerating neuronal CA1 layer. These changes were modified by D-JNKI1. We may hypothesize that D-JNKI1 exerts a neuroprotective effect directly on neurons (shown in this *in vitro* study by localization of D-JNKI1 in NeuN-positive cells and by previous data in neuronal cultures, (Borsello et al., 2003), and modifies the cellular signaling from affected neurons to microglia. Thus, D-JNKI1 would act indirectly on microglia through signals secreted by neurons influencing microglial activity and localization.

Interestingly, the hippocampal slice hypoxia-hypoglycemia model may not reproduce mechanisms of microglial activation observed in the *in vivo* stroke model. Indeed, D-JNKI1 showed no effect on microglial activation and accumulation at 48 h after MCAO, while changes in morphology and localization were observed *in vitro*. This may reflect species, age differences (adult mice used *in vivo* versus newborn rats *in vitro*) and different concentration of the inhibitor, which might cause discrepancies. Furthermore, *in vitro* models are only partially representative of the *in vivo* situation concerning the microglial response. Indeed, we saw DCs and infiltration of inflammatory cells, such as T lymphocytes after MCAO (Figure 9), which evidently does not occur *in vitro*. These cells could modulate microglial activation and may explain the differences seen in the two models. Besides the activation and accumulation of glial and inflammatory cells in the brain parenchyma are time dependent (Gelderblom et al., 2009). Thus *in vivo* we might not observed an effect on microglial activation 48 h after the injury, but probably we could found changes at later time as the inflammatory response in the tissue last for several days (Dirnagl, 1999). It is worth noting that in a complex situation such as brain inflammation following stroke which depends of several paramaters (time, type of cells, localization), it would not be suprising that we obtained different results in our models.

4.5 CONCLUDING REMARKS OF THE 1ST PART

Here we demonstrated that D-JNKI1, a selective JNK inhibitor and highly neuroprotective peptide, does not attenuate the activation and accumulation of microglia in the ischemic tissue in an *in vivo* MCAO model of stroke at 48 h. This finding adds to the knowledge that neuroprotection via inhibition of JNK is independent of microglial accumulation or activation. It also indicates that the JNK pathway might be not essential to microglial activation after ischemia, and that the presence of activated microglia in the ischemic area is not necessarily detrimental to neurons. Additionally, our study suggests that D-JNKI1 could be safely used in clinical stroke patients, as it does not exacerbate inflammatory responses after cerebral ischemia. Our *in vitro* data point towards an effect of D-JNKI1 on microglial recruitment as well as morphological changes, which is likely to be an indirect effect of the peptide through its action on neurons.

There are many controversies about the protective or detrimental role of microglia on neuronal survival after cerebral ischemia, namely whether activated microglia are enhancing or reducing neuronal damages. At this point, we can assume that the activation of microglia is not detrimental. However, investigating the morphology of microglia is not sufficient to completely rule out this hypothesis. Therefore, looking at the pattern of secreted pro- and anti-inflammatory mediators in brain ischemia with and without D-JNKI1 treatment will help to characterize the role of inflammation during cerebral ischemia. Furthermore, as D-JNKI1 was not detected in the brain by immunohistochemistry, it could also possibly inhibit peripheral JNK activation, which could lead to a modulation of peripheral inflammatory processes and indirect neuroprotection. The second part of the discussion will address this issue.

SECOND PART: EFFECT OF NEUROPROTECTION ON THE SECRETION OF INFLAMMATORY MEDIATORS

Most inflammatory reactions are mediated by the secretion of cytokines and chemokines, such as for instance upregulation of adhesion molecules, recruitment and stimulation of leukocytes, activation of glial cells, induction of intracellular inflammatory signaling pathways (Wang et al., 2007). An increase of pro- and anti-inflammatory mediators has been reported in experimental models of cerebral ischemia as well as in patients with acute stroke. The association between inflammatory mediators and brain damage as well as stroke progression and prognosis is not well defined. Many discrepancies actually exist among several investigations both in animal stroke models and clinical studies. These contradictory results have been discussed in the second part of the Introduction. Thus, there is a great need to understand the inflammatory processes after stroke and whether they lead to tissue repair or damage, in the purpose of better targeting drugs or of avoiding research on the wrong target. Taking advantage of our mouse model of potent neuroprotection following cerebral ischemia, we investigated the systemic and local brain inflammatory response in order to better characterize the role of post-stroke inflammation and to assess whether cytokine/chemokine modulation is dependent on the JNK signaling pathway. Regarding the potential regulation of IL6 and KC by the JNK pathway (see Introduction, sections 1.5.3 and 1.5.4) and based on recent findings that these two mediators displayed the most significant changes in release following cerebral ischemia (Chapman et al., 2009), we chose to analyze the secretion of these two inflammatory molecules in the systemic circulation and in the brain following experimental cerebral ischemia. Our concluding remarks are therefore extrapolated from our results on these two inflammatory mediators.

4.6 SYSTEMIC INFLAMMATION FOLLOWING STROKE

It has now been well described that a systemic increase of cytokine and chemokine levels occurs very early following stroke. In accordance with previous clinical and

experimental studies, we found a significant plasma release of IL6 and KC between four and seven hours after the onset of cerebral ischemia compared to sham mice (Figure 19), (Yamasaki et al., 1995; Emsley et al., 2003; Offner et al., 2006; Chapman et al., 2009). The chemokine CXCL1/KC is thought to generate an environment which could induce a gradient and favor leukocyte recruitment to the brain and potentiate the ischemic damage (see Introduction, sections 1.5.2 and 1.5.4). The implication in the lesion development of the early systemic IL6 secretion following stroke, despite extensive studies, is far from clear (see Introduction, section 1.5.3). It is thus not well established whether the increase of systemic inflammatory mediators plays a pathogenic part in cerebral ischemia, reflects the ischemic brain injury or has other physiological roles. Taking advantage of the established neuroprotective effect of D-JNKI1 in experimental mouse models of cerebral ischemia (Wiegler et al., 2008; Benakis et al., 2010), we assessed the secretion of IL6 and KC in the plasma of mice injected intravenously with a single dose of D-JNKI1, 3 h after the onset of ischemia. Surprisingly, we found no significant difference neither in early systemic IL6 release nor KC release between the vehicle and D-JNKI1-treated groups, even though we measured a significant decrease by half of the lesion volume 48 h after MCAO (Figure 22). These interesting findings reveal that the early systemic release of IL6 and KC is not correlated with an unfavorable outcome. Thus, the early increase of systemic inflammatory mediators following cerebral ischemia is not a relevant biomarker for poor prognosis, at least in our model.

Even though we showed that early systemic inflammation following stroke is independent of poor outcome, it would be of great interest to understand the causes and consequences of this increase of plasma cytokine/chemokine secretion, as for instance investigating their source of production. We assessed the secretion of both these inflammatory mediators also in the ischemic brain at several time points. In accordance with others, we found no significant difference of both IL6 and KC brain release at four and seven hours between MCAO and sham mice (Figure 22), (Chapman et al., 2009). Because we measured an increase of cytokine/chemokine early in the plasma and not in the brain, thus we assumed that the brain was not the main source of early systemic

inflammation. Offner and colleagues found an increased release of several cytokines from activated and unstimulated mouse splenocytes 6 h after cerebral ischemia (Offner et al., 2006). Chapman et al. found no induction of IL6 in lungs and liver after experimental stroke, whereas hepatic and pulmonary expression of CXCL1 was elevated in correlation with the systemic temporal profile (Chapman et al., 2009). Thus, early after ischemia, peripheral organs are able to secrete inflammatory mediators. Altogether, we hypothesize that the peripheral immune system is the main source of early systemic inflammation, and probably acts as an early response reflecting the ongoing injury rather than a mechanism leading to an increase in cerebral damage: firstly, because we obtained neuroprotection despite early systemic inflammation; secondly, because systemic inflammation was transient, indeed we reported an increase of IL6 and KC in the plasma until 7 h after ischemia and a drastic decrease between 24 h and 48 h, especially for the non-D-JNKI1-treated group (Figure 23).

How does cerebral ischemia result in an early systemic inflammatory response in the absence of a local significant cerebral inflammation? There are several axes of communication between the central nervous system and peripheral immune system: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS), (Elenkov et al., 2000; Pedemonte et al., 2006). Thus, within minutes of the occlusion, signals from the damaged brain could be sent to the periphery and activate an immune response which would drive the secretion of inflammatory mediators. It has to be noted that, even though our data suggest that there is no detrimental effect of the early systemic inflammation, we still need to understand why a massive activation of the peripheral immune system takes place early after brain injury.

We have started to examine the effect of D-JNKI1 on the spleen, liver and lungs. Preliminary results indicate a decrease of IL6 release from the spleen at 7 h in mice treated with D-JNKI1 and not in vehicle-treated mice, in which this decrease takes place later. Thus we propose that D-JNKI1, by reducing neuronal death, might indirectly restrain a sustained peripheral inflammatory response which could be unfavorable for the late outcome. This issue clearly needs to be further examined.

4.7 BRAIN INFLAMMATION FOLLOWING STROKE

Release of IL6 and KC was investigated in the brain of vehicle and D-JNKI1 groups at different time points. We could not detect a significant increase of KC in the brain at any time point compared to sham mice, contrasting with results published by other groups (Chapman et al., 2009; Yamasaki et al., 1995). This difference could have several explanations: 1) Chapman et al. performed a longer occlusion duration (60 min occlusion, V = 67 mm^3), which could explain the increase of KC in their model; 2) different animal species were used, which may display different inflammatory reactions in response to cerebral ischemia; 3) different anesthetic agents were used, and the anesthesia itself might modulate the production of inflammatory mediators (Denes et al., 2010b). We found a significant increase of brain IL6 release at 48 h between sham and MCAO mice. However, D-JNKI1 did not reduce IL6 secretion at 48 h. Suzuki and colleagues suggest that IL6 may contribute to both injury and repair during the acute phase of cerebral ischemia. However the peak of IL6 expression would be associated with neuroprotection (Suzuki et al. 2009). We assume that brain inflammation mediated by IL6 is not detrimental at 48 h, as we found a significant reduction of the lesion volume of mice treated with D-JNKI1 (Figure 22). Furthermore, we carefully recorded the parameters (temperature, weight and neuroscore listed in Appendix: Tables 2) at sacrifice in all three groups. Forty-eight hours after injury, the weight of treated mice used in the ELISA study was significantly higher than that of vehicle mice, which shows a better well-being of treated mice at this time point and confirms the neuroprotection in this separate set of experiments. It is worth noting that we used a moderate model of MCAO (transient occlusion for 30 minutes), which commonly leads to striatal and cortical lesions or, in some animals, to a striatal lesion only. The variability of the lesion size in this model could explain that we do not measure a significant difference of cytokine/chemokine release between groups.

Another interesting issue that needs to be discussed is the survival analysis after MCAO in both groups. We noticed that, even though the survival prognosis showed no overall significant difference between groups, no death was recorded from 24 h after the injury onwards in the D-JNKI1-treated group compared to the vehicle group

(Appendix, Table 4). The survival proportion in the vehicle group starts to decrease to 65% at 4 days and reaches 55% at 5 days, whereas that of the D-JNKI1 group remains stable until 5 days (91%). Therefore, we assume that D-JNKI1 may have an effect on IL6 release at late time points, especially on the plasmatic concentration. Indeed, we observed a trend towards a reduction of IL6 concentration in the plasma and slightly in the brain of treated animals five days after the injury (Figure 22, $p_{plasma} = 0.27$, $p_{brain} = 0.42$). However, this potential reduction of IL6 release might not be apparent in our data because of the higher proportion of death at 48 h and 5 days in vehicle mice than in treated mice. Overall, we can suppose that mice with a better prognosis would have a reduced inflammatory response at late time points. This effect might be mediated by the neuroprotective effect of D-JNKI1. Thus, we suggest that systemic and brain inflammation at late time points are a consequence of the neuronal damage and could favor poor outcome, but are not the direct cause of the injury. This hypothesis needs further investigation.

4.8 REGULATION OF THE JNK PATHWAY AND SECRETION OF INFLAMMATORY MEDIATORS

The regulation of IL6 and IL8 (KC/CXCL1) expression, among other inflammatory mediators, is partly mediated by the activation of the JNK intracellular signaling pathway, as these genes contain an AP-1 site in their promoter region (Waetzig et al., 2005). JNK is activated during cerebral ischemia, therefore we assumed that D-JNKI1 would modulate the secretion of both cytokines through the inhibition of the JNK pathway. Furthermore, the peptide was injected intravenously; thus, we could expect a peripheral effect on cytokine secretion, as JNK is expressed ubiquitously. However, as described above, we did not find any significant difference in the level of brain and systemic IL6 and KC secretion following ischemia between groups treated with D-JNKI1 or not. Nijboer and colleagues evaluated the effect of a short half-life inhibitor of JNK, L-JNKI1, on the mRNA expression of several cytokines and chemokines in a model of perinatal hypoxia–ischemia (HI). The inhibition of the JNK/AP-1 pathway did not reduce brain cytokine/chemokine expression 3 h after induction of HI, even

though the peptide was neuroprotective. We found similar results on the *de novo* brain synthesis of inflammatory mediators using a JNK inhibitor with a longer half-life. Thus, neither the mRNA expression nor the secreted cytokine/chemokine are down-regulated by the inhibition of JNK (Nijboer et al., 2010). These results strongly suggest that the JNK pathway, which leads to the synthesis of inflammatory mediators, is not affected by JNK inhibitors after cerebral ischemia. Furthermore, these findings suggest that not all JNK targets are inhibited by L- or D-JNKI1. This in itself is of great interest, as it would be detrimental to totally inhibit the JNK pathway, since it has diverse physiological functions that might be necessary for cell survival.

4.9 CONCLUDING REMARKS OF THE 2ND PART

Here we demonstrated that D-JNKI1, a highly neuroprotective peptide, does not attenuate the release of IL6 and KC in the systemic circulation early after the ischemic injury. This very interesting finding adds to the knowledge that increased plasma levels of inflammatory mediators are not a biomarker for an irreversible brain lesion. Several clinical and experimental studies report an increase of systemic inflammatory mediators after stroke and an association with an unfavorable outcome. Indeed, in vehicle mice subjected to MCAO, we noticed at first that systemic inflammation precedes brain inflammation and, importantly, the onset of the visible lesion in the brain parenchyma (Figure 21). Thus, a linear relationship of cause and effect could easily be inferred: the systemic inflammation increases the brain injury. It may be for this reason that much experimental research focused on this early post-stroke process to target neuroprotective drugs. However, our results clearly indicate that neuroprotection occurs despite the early increase of systemic inflammation. Thus, there is no point in therapeutic drugs targeting this early event. Furthermore, we found a trend towards a reduction of the systemic concentration of inflammatory mediators as late as five days after ischemia in mice treated with D-JNKI1 as well as a tendency towards better survival. Therefore, we suggest that the systemic increase of inflammatory mediators reflects the ongoing injury, particularly at late time points, and might have a consequence on the overall impairment of the patient's condition. In addition, we

showed that *de novo* cytokine secretion in the brain did not decrease after neuroprotective treatment, while we observed a reduction of the lesion volume.

Together with our results described in the first part of this project, we showed that neuroprotection occurs independently of microglial activation and cytokine release in the brain and systemic circulation. Besides, the inhibition of the JNK pathway, one of the main regulators of inflammatory genes transcription, is not sufficient to inhibit inflammatory mediator secretion and microglial activation.

5. CONCLUSION AND PERSPECTIVES

Throughout the past twenty years, the brain inflammatory reaction to an ischemic stroke has been carefully examined. Nowadays it is incontestable that inflammatory cells and mediators are present in and around the ischemic brain tissue. However, the beneficial or detrimental role of inflammation during cerebral ischemia is still hotly debated. This project aimed at better characterize the highly regulated post-stroke inflammation process.

The first key result of this work is that neuroprotection occurs even though there is inflammation following experimental stroke. Indeed, after cerebral ischemia, we observed in the brain parenchyma that microglial cells are activated, cytokines and chemokines are secreted independently of the reduction of the neuronal death. In the systemic circulation, level of inflammatory mediators is elevated early after ischemia regardless the outcome. Our second important result is that inhibition of the intracellular JNK signaling pathway using the highly neuroprotective peptide D-JNKI1 does not influence directly post-stroke inflammation. Altogether, we report that inhibition of inflammation is not necessarily required to obtain a high degree of neuroprotection after cerebral ischemia. Thus, post-stroke inflammation might not be detrimental for the tissue recovery.

These findings may be seen as negative results because they were not what we expected, namely we did not obtain inhibition of inflammation in a model of experimental cerebral ischemia as we first postulated in our hypothesis. Editors and even authors are not always eager to publish negative results, which may be seen as less interesting. However, it is a mistake not to report studies that are negative but cautiously designed and which lead to interpretable results. In this regard, we need to address the fact that our negative results could be explained by two important aspects that need to be mentioned. First, our MCAO model was carried out in outbred mice. This is a very relevant strain for clinical issues because these mice represent a human population better than inbred mice, which are genetic clones. However, the use of non-

consanguineous mice leads to a potentially higher degree of variability, and we are aware that it could potentially hide the differences between treatment groups. Second, inflammatory processes and intracellular signaling pathways are highly regulated and redundant mechanisms. This means that shutting down one pathway, one type of inflammatory cells or one cytokine or chemokine would not necessarily induce a significant phenotype because of compensating mechanisms. In this respect, we could increase the number of animals per group to see if we observed a difference between groups. Furthermore, it would be interesting to inhibit several intracellular signaling pathways that could have a more pronounced effect on the secretion of inflammatory mediators. Overall, carefully performed experimental studies which lead to either positive or negative results bring us closer to a better understanding of the disease and of its underlying cellular and molecular mechanisms, to an improvement of the potential treatments and of the way to test them in clinical trials. Even though negative results may not be the final step toward a new treatment, they certainly help researchers to raise new hypotheses.

Despite the fact that we found interesting 'negative' results, this project is far from having answered all the questions about the role of inflammation after stroke and, in our case, the effect of the JNK inhibitor D-JNKI1 on inflammation. Many routes still need to be investigated. In this section, we propose experiments which could lead to a better understanding of the regulation of the JNK pathway and of the inflammatory process. We have mentioned (Introduction, section 1.5.3) that activation of JAK/STAT pathway was induced by IL6 binding to its associated receptor, leading to a series of phosphorylation events, which stimulates the transcriptional activities of Stat3. Activated Stat3 induces the expression of the SOCS3. The suppressor of cytokine signalling (SOCS) proteins have a broad range of effects, including the modulation of cytokine signaling in various diseases of the CNS (Baker et al., 2009). In experimental models of cerebral ischemia, SOCS3 expression is up-regulated. In addition, antisense knockdown of SOCS3 protein expression increased the lesion volume, suggesting a neuroprotective role of SOCS3 (Bates et al., 2001; Raghavendra Rao et al., 2002). Interestingly, Stat3 can undergo phosphorylation at several sites, some of which may

lead to an inhibition of its transcriptional activity. For instance, activation of the JNK pathway inhibits Stat3 DNA binding and its transcriptional activities after stimulation by IL6 (Lim & Cao, 1999; Schuringa et al., 2000; Heinrich et al., 2003). Thus, the JNK pathway appears to negatively regulate Stat3 and therefore probably also SOCS3. We can hypothesize that when JNK is inhibited by D-JNKI1, Stat3 would be up-regulated and induce SOCS3 expression, which could lead to neuroprotection via cytokine modulation. According to this hypothesis, we propose to analyze the phosphorylation state of Stat3 in our MCAO model with or without D-JNKI1. We could therefore obtain a new pathway of the regulation of the JNK pathway by D-JNKI1 stimulated by IL6. Altogether, we hypothesize that p-Stat3 activation mediated by IL6 through the JAK/STAT pathway as well as the inhibition of the JNK pathway could be associated with neuroprotection. Figure 24 illustrates this hypothesis on the intracellular pathways that could be regulated by D-JNKI1.

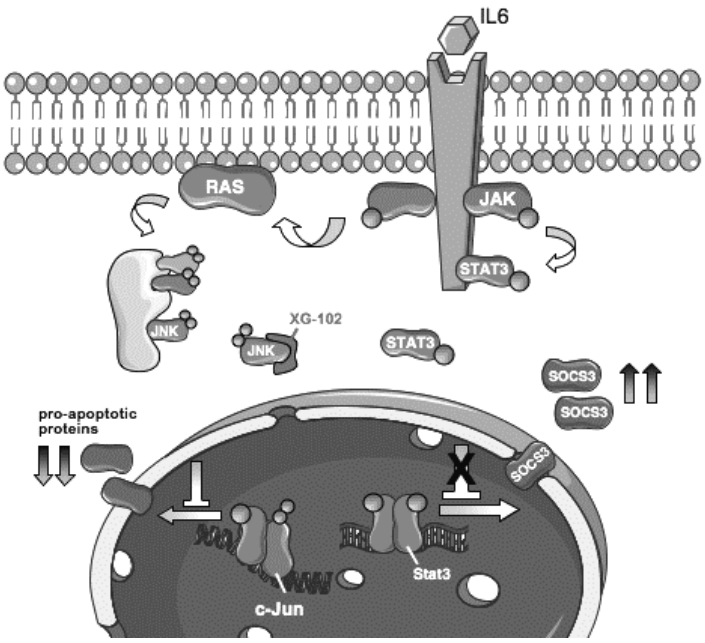


Figure 24. Hypothesized effect of D-JNKI1 on the intracellular pathway activated by IL6

On the left part, we show the effect of D-JNKI1 inhibition on the AP1 complex transcription activity. On the right part, we illustrate the activation of the JAK/STAT pathway. We hypothesize that D-JNKI1, by inhibiting JNK, could up-regulate Stat3 and lead to the transcription of SOCS3, which could be neuroprotective in MCAO models. (Figure was produced using Servier Medical Art).

This proposed experiment illustrates that D-JNKI1 could have an effect on post-stroke inflammation through modulation of intracellular pathways. However based on results of the present work, the direct link between inflammation and inhbition of JNK in our experimental models is not yet elucidated. In the state of our current knowledge we

suggest that D-JNKI1, by reducing the neuronal death, could ameliorate the overall condition of the subject and would indirectly modulate at late time points the inflammation of peripheral organs and probably also inflammatory mechanisms in the brain tissue. Therefore, we would like to investigate the secretion of inflammatory mediators after MCAO in the plasma and in the brain, spleen, liver and lungs at later time points (such as 7 days and 14 days) in vehicle and treated animals. Additionally, information of the inflammatory profile in the lungs after stroke would be interesting as we could investigate the phenomenon of stroke-induced 'immunosuppression' and the occurrence of pneumonia, and whether D-JNKI1 inhibits this unfavorable outcome.

We have seen that inflammation after cerebral ischemia is a very complex mechanism which is far from being understood, as many discrepancies between experimental findings exist. I think that controversial ideas on the beneficial and especially the detrimental role of post-stroke inflammation could stem from preconceived notions. Indeed, in other diseases of the CNS such as multiple sclerosis, cerebral damage is universally thought to be a direct consequence of brain inflammation. Therefore, cerebral inflammation comes to be seen as uniformly detrimental. However, in the context of stroke, I propose that inflammation occurring in the brain may be better seen as necessary to rescue the damaged tissue and even to mediate tissue repair. It would therefore not exacerbate the neuronal death, but on the contrary limit it and promote recovery. Furthermore, I deem essential to better appreciate the distinctive temporal and spatial contributions of systemic and brain inflammation following stroke. I suggest that early systemic inflammation is the reflect of the ongoing injury, then that brain inflammation is activated to clear the damaged tissue, and later that systemic and brain inflammation mirror the general condition of the subject. Despite the discouraging of clinical trials of neuroprotection so far, I suggest that it is of great interest for patients to continue the development of therapeutic drugs based on neuroprotective agents, because they could prevent neuronal death and indirectly late inflammatory processes, which altogether would be greatly beneficial for a better recovery after stroke.

6. APPENDIX

Tables 2 (Figures 19, 20, 22C, 22D, 23)

MCAO 30 min – Sacrifice at 4 h, 7 h, 24 h, 48 h, 5d – Cytokine assay

Results are presented as mean ± SEM, except for neurological score expressed as [median, min, max]. *p<0.05 indicates a significant difference in MCAO+vehicle mice versus MCAO+D-JNKI1 mice. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ indicate a significant difference between sham mice and MCAO (vehicle or D-JNKI1) mice. There was a better reperfusion in the D-JNKI1 treated group sacrified at 7 h (*p < 0.05). In the group sacrified at 48 h, weight at sacrifice was lower in vehicle- and D-JNKI1-treated mice than sham mice ($^{\#\#\#}p < 0.001$ and $^{\#\#}p < 0.01$, respectively). Furthermore, there was a less weight loss in the D-JNKI1 group than the vehicle group (*p < 0.05). At 5 d, weight at sacrifice of vehicle- and D-JNKI1-treated mice was lower than sham mice ($^{\#}p<0.05$).

4h	Sham (n = 5)	Vehicle (n = 6)	D-JNKI1 (n= 7)
CBF during ischemia (%)	-	11.0 ± 1.2	13.0 ± 1.1
CBF after ischemia (%)	-	67.5 ± 6.5	67.9 ± 8.3
Weight (g)	28.6 ± 2.1	28.2 ± 1.7	27.9 ± 1.0
Weight at sacrifice (g)	27.6 ± 1.6	26.5 ± 1.7	26.4 ± 1.2
T during ischemia (°C)	36.5 ± 0.2	36.7 ± 0.06	36.6 ± 0.1
T at sacrifice (°C)	36.1 ± 0.5	35.5 ± 0.3	35.1 ± 0.1
Neuroscore at 4 h	-	2.0, 1.0, 3.0	1.75, 1.0, 2.0

7h	Sham (n = 5)	Vehicle (n = 10)	D-JNKI1 (n= 7)
CBF during ischemia (%)	-	11.6 ± 1.4	10.9 ± 0.9
CBF after ischemia (%)	-	57.0 ± 2.9	76.4 ± 6.9*
Weight (g)	30.4 ± 1.7	29.6 ± 0.9	28.9 ± 1.7
Weight at sacrifice (g)	29.7 ± 1.7	28.1 ± 1.0	26.9 ± 1.7
T during ischemia (°C)	36.6 ± 0.1	36.8 ± 0.05	36.6 ± 0.2
T at sacrifice (°C)	36.1 ± 0.2	35.9 ± 0.4	35.4 ± 0.2
Neuroscore at 7 h	-	2.0, 1.5, 3.0	1.5, 1.0, 3.0

24h	Sham (n = 3)	Vehicle (n = 6)
CBF during ischemia (%)	-	13.7 ± 1.6
CBF after ischemia (%)	-	69.2 ± 8.6
Weight (g)	28.3 ± 2.8	28.7 ± 1.9
Weight at sacrifice (g)	28.0 ± 2.0	23.8 ± 1.7
T during ischemia (°C)	36.6 ± 0.2	36.8 ± 0.1
T at sacrifice (°C)	36.0 ± 0.7	35.4 ± 0.4
Neuroscore at 48 h	-	1.25, 1.0, 2.0

48h	Sham (n = 7)	Vehicle (n = 9)	D-JNKI1 (n = 8)
CBF during ischemia (%)	-	12.0 ± 1.2	12.5 ± 2.3
CBF after ischemia (%)	-	60.3 ± 4.3	62.6 ± 2.7
Weight (g)	27.9 ± 1.1	28.0 ± 0.7	30.8 ± 1.0
Weight at 24 h (g)	27.3 ± 1.2	23.0 ± 0.5	26.0 ± 0.9
Weight at sacrifice (g)	29.0 ± 0.9	21.4 ± 0.6###	24.6 ± 1.2##/*
T during ischemia (°C)	36.4 ± 0.2	36.5 ± 0.1	36.5 ± 0.06
T at 24 h (°C)	35.4 ± 0.3	35.0 ± 0.3	35.0 ± 0.2
T at sacrifice (°C)	35.6 ± 0.3	34.4 ± 0.3	34.3 ± 0.4
Neuroscore at 24 h		1.5, 1.0, 2.0	1.75, 1.0, 2.0
Neuroscore at 48 h	-	1.5, 1.0, 2.0	1.5, 1.0, 3.0

5d	Sham (n = 8)	Vehicle (n = 6)	D-JNKI1 (n = 7)
CBF during ischemia (%)	-	15.3 ± 2.0	9.8 ± 1.4
CBF after ischemia (%)	-	63.3 ± 5.7	63.1 ± 6.6
Weight (g)	28.1 ± 1.3	29.7 ± 1.4	28.9 ± 0.8
Weight at sacrifice (g)	30.3 ± 1.4	24.5 ± 2.0#	23.4 ± 1.4#
T during ischemia (°C)	36.3 ± 0.1	36.9 ± 0.2	36.7 ± 0.1
T at sacrifice (°C)	35.7 ± 0.2	34.7 ± 1.0	34.7 ± 0.3
Neuroscore at 48 h	-	1.25, 1.0, 1.5	1.0, 0, 1.5

Table 3 (Figure 22A)

MCAO 30 min – 48 h reperfusion – PFA fixation

Results are presented as mean ± SEM, except for neurological score expressed as [median, min, max].

	Vehicle (n = 7)	D-JNKI1 (n = 5)
CBF during ischemia (%)	11.3 ± 2.0	9.4 ± 1.2
CBF after ischemia (%)	61.6 ± 5.5	57.4 ± 6.0
Weight (g)	29.6 ± 0.6	29.6 ± 0.9
Weight at sacrifice (g)	22.7 ± 0.9	23.2 ± 1.0
T during ischemia (°C)	36.6 ± 0.1	36.6 ± 0.06
T at sacrifice (°C)	34.7 ± 0.4	34.5 ± 0.5
Neuroscore at 48 h	1.5, 1.5, 2.0	1.0, 1.0, 1.5

Table 4 (Figure 22B)

Survival proportions [%] of mice in the vehicle group or treated with D-JNKI1.

Hours [min]	Vehicle (n = 57)	D-JNKI1 (n = 45)
0.000	100.000	100.000
4.000	98.24561	100.000
5.000	96.2807	
6.000	94.31579	
7.000	94.31579	100.000
24.000	91.83379	90.625
48.000	91.83379	90.625
96.000	64.28366	
120.000	55.10027	90.625

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Inflammation and stroke[1]

Corinne Benakis[a], Lorenz Hirt[a], Renaud A. Du Pasquier[a, b]

[a] Service of Neurology, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne

[b] Service of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne

Summary

It is well-known that inflammation plays a major role in the genesis of the atherosclerotic plaque and thus favours the occurrence of stroke. But, inflammation is also involved after an ischaemic event affecting the brain. In this case, one can observe the infiltration of numerous inflammatory cells at the site of the lesion, the activation of the microglia and of pro-inflammatory cytokines, etc. It is usually thought that this post-stroke inflammation is rather deleterious, as suggested by the fact that, after an experimentally-induced ischaemic stroke, blockade of the inflammatory response improves the neurological condition of mice. Nevertheless, until now, the application of such experimental treatments in humans has revealed unsuccessful. One of the possible explanations for this phenomenon might be that inflammation also has some beneficial effects, such as clearance of damaged tissue, promotion of angiogenesis, or still tissue remodelling and regeneration.

After this first "basic science" part, we will briefly review some clinical aspects of the most significant inflammatory diseases that can cause stroke, i.e., the vasculitis. Among them, Takayasu's arteritis, giant cell temporal arteritis (Horton's disease), and primary angiitis of the central nervous system will be discussed. We will also shortly address the question of the antiphospholipid syndrome.

Key words: cytokines; ischaemia; microglia; neuroprotection; vasculitis.

Inflammatory mechanisms related to an ischaemic stroke

Brain ischaemia

Stroke is a leading cause of long-term disability and remains the third cause of death in developed countries (World Health report 2007, World Health Organisation). While haemorrhagic stroke triggers cerebral oedema and inflammation, this short review focuses on the more common ischaemic stroke. Importantly, while

Figure 1

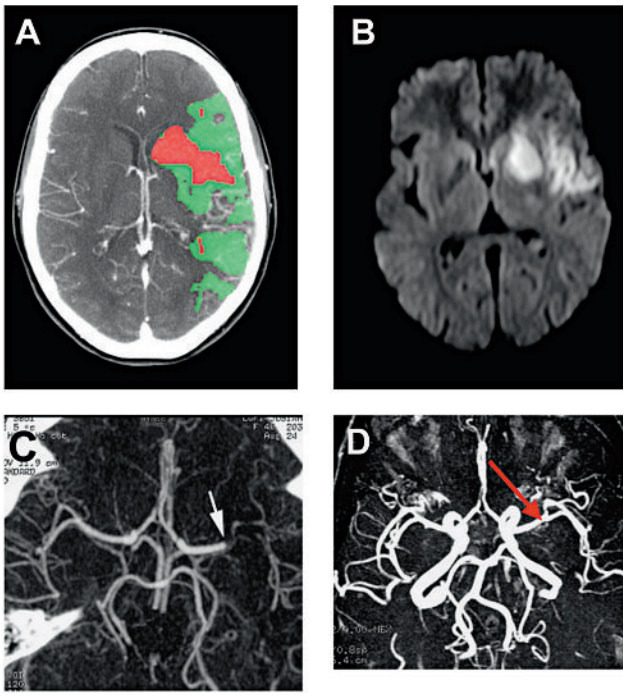
Ischaemic stroke in the left middle cerebral artery.

A Perfusion cerebral computerised tomography (CT) shows an ischaemic core (red) surrounded by penumbra (green) [52]. The core corresponds to the irreversible ischaemic insult (infarction), whereas the penumbra represents brain parenchyma which is suffering from ischaemia but which can be rescued if the blood perfusion is rapidly restored.

B The diffusion sequences on brain MRI show a hyperintensity in the territory of the left middle cerebral artery. Of note, only the infracted territory, which corresponds to the core in (A) is visible.

C Angiographic sequences of cerebral CT show a sub-occlusion of the left middle cerebral artery (arrow).

D The patient benefited from an intra-venous thrombolysis with recombinant tissue plasminogen activator (rtPA). Few days later, angiographic sequences on brain MRI showed a complete repermeabilisation of the left middle cerebral artery, which paralleled a favourable clinical outcome. (With courtesy of Dr A. Croquelois and the Division of Neuroradiology, Lausanne, Switzerland.)



Correspondence:
Renaud A. Du Pasquier, Assistant professor
Service of Neurology
Centre Hospitalier Universitaire Vaudois (CHUV)
46, rue du Bugnon
CH-1011 Lausanne
Renaud.Du-Pasquier@chuv.ch

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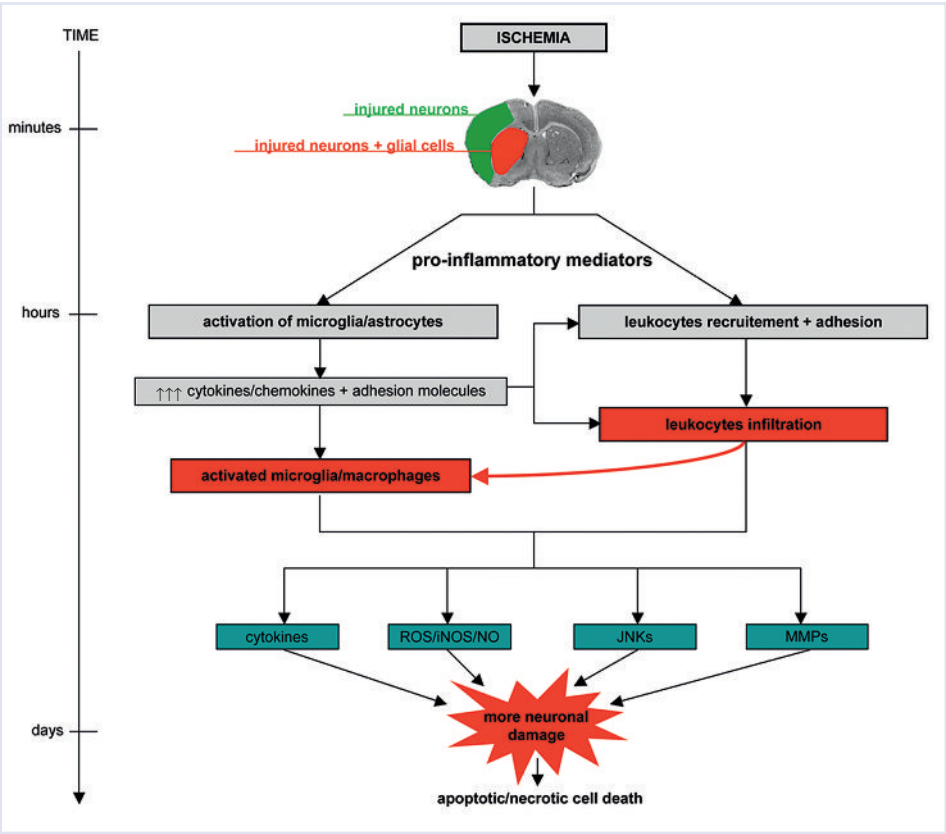
ischaemia at first induces only a loss of function at its very early stages, structural damage appears rapidly thereafter and progresses as minutes and hours go by. Reduction of cerebral blood flow leads to a lack of oxygen and glucose supply to the brain parenchyma. This nutrient deficiency triggers multiple events including a dramatic depletion of ATP, perturbation of the cellular ionic homeostasis, neurotransmitter release and activation of many cytotoxic enzymes. The release of the excitatory neurotransmitter glutamate leads to excessive excitotoxic stimulation of glutamate receptors in energy deprived neurons. Excitotoxicity is a major mechanism in the early stages of the progression of ischaemic brain injury. Other detrimental events include peri-infarct depolarisation, apoptosis and inflammation (fig. 1) [1, 2].

Several therapeutic strategies aimed at decreasing the effect of these ischaemia-induced phenomena have been successful in animal stroke models, but not so far in stroke patients. The only successful treatment in the acute phase for stroke patients is thrombolysis, the goal of which is to restore the blood flow to the brain [3, 4]. As it needs to be administered intra-venously within 3 hours to 4.5 hours or intra-arterially within 6 hours from symptom onset it is limited to a small number of patients [5]. Beyond this time-window restoring cerebral blood flow is no longer beneficial and there is an increased risk of developing a symptomatic intracerebral haemorrhage [6]. Furthermore, the recombinant tissue plasminogen activator (rtPA) enhances excitotoxicity and increases the lesion volume in our mouse MCAo model [7, 8]. Thus, there is a need for new treatments for the patients who can not be thrombolysed.

An important delayed mechanism beginning within hours from the onset of ischaemia is the robust inflammatory response in the ischaemic tissue. There is increasing evidence showing a detrimental effect of the post-ischaemic inflammatory reaction [9, 10]. Therefore therapeutic strategies targeting the delayed inflammatory response could inhibit the progression of the tissue damage providing an extended therapeutic window for neuroprotection.

Figure 2

Diagram showing inflammation after stroke. Within minutes after cerebral ischaemia excitotoxic mechanisms are activated in neurons in the territory of the occluded artery which lead to apoptotic cell death independently of inflammatory mediators. These injured neurons both in the core (shown in red) and periphery (shown in green) of the lesion and glial cells in the core are producing pro-inflammatory mediators (such as cytokines and reactive oxygen species) which highly activate glial cells, leading to more cytokines and chemokines being released from activated microglia and astrocytes, and to an upregulation of adhesion molecules in vascular endothelial cells. Adhesion molecules and chemokines mediate the recruitement of circulating leukocytes to the vessel wall. Their infiltration or diapedesis into the ischaemic tissue occurs via highly specific receptor-ligand interactions between endothelial cells and leukocytes. In the next few hours after injury, once activated in the parenchyma, leukocytes and microglia (red boxes) produce more inflammatory mediators such as cytokines, nitric oxide (NO) through inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS) and metalloproteinases (MMPs). Stress signalling pathways (JNKs) are also up-regulated and induce transcription of cytotoxic and inflammatory genes (blue boxes). This local inflammatory response contributes to secondary injury to potentially viable tissue and leads within hours and days to apoptotic or necrotic neuronal cell death (adapted from [12]).



Neuroinflammatory response after ischaemia

The brain immune defence system is essential to protect neurons against infectious agents. In cerebral ischaemia, the inflammatory response plays a role in the clearance of cell debris; however it also enhances the damage to the tissue.

An immune response at the site of injury is characterised by the infiltration, accumulation and activation of inflammatory cells. Within hours after the onset of focal cerebral ischaemia peripheral leukocytes adhere to the cerebral endothelium, cross the vessel wall and invade the damaged parenchyma [11, 12]. At the same time astrocytes and microglia be-

come activated. These cellular events depend on the secretion of inflammatory mediators which are produced by neural and glial cells in response to an ischaemic insult. Once activated in the site of injury inflammatory cells start to secrete a large variety of cytotoxic agents such as cytokines, chemokines and promote the expression of adhesion molecules, matrix metalloproteinases (MMPs) with an increased production of free radicals. Stress signalling pathways, such as the c-Jun N-terminal kinases (JNKs) pathway are also activated. Both the JNK pathway and pro-inflammatory mediators further potentiate the brain tissue injury and lead within hours and days to apoptotic and necrotic cell death of the potential viable tissue [13–15]. Figure 2 provides a schematic diagram illustrating the inflammatory response after ischaemic stroke.

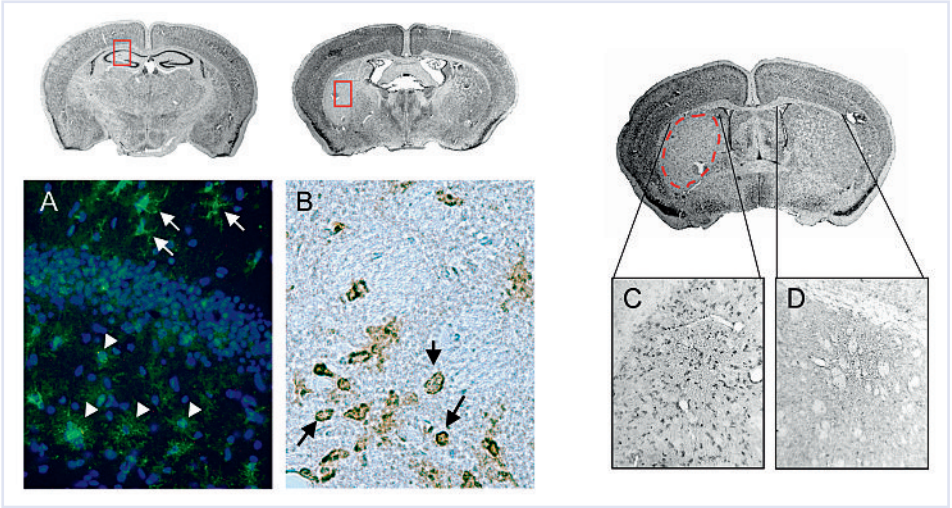
Table 1
Classification of different vasculitis types.

Vasculitis	
Primary	**Secondary**
Large-size arteries	Systemic lupus erythematosus ± anti-phospholipid syndrome
Giant cell temporal arteritis (Horton)	Behçet disease
Takayasu arteritis	Sjögren syndrome
Middle-size arteries	Neuro-sarcoidosis
Primary angiitis of the central nervous system	Rheumatoid arthritis
Polyarteritis nodosa	Scleroderma
Small-size arteries	Inflammatory bowel diseases
Churg Strauss	Infections, e.g., varicella-zoster virus vasculopathy
Wegener	Others
Microscopic polyarteritis	

Glial cell activation

Microglia are the resident macrophages of the brain. They are very sensitive to subtle alterations in their neuronal microenvironment. Resting microglia has a very ramified cytoplasm, covering a territory of 30–40 µm in diameter. In response to an injury, they quickly become activated and undergo morphological transformations as well as functional changes. They start to retract their long processes and their shape becomes rounded – so called phagocytotic, amoeboid microglia [16–18]. The degree of microglial activation depends on the severity of neuronal injury. The mildest injuries may only cause ramification of microglia with a bushy appearance, whereas in acute pathological cases microglia are characterised by hypertrophic bodies and less arborised processes. If neurons die, microglia transform into brain macrophages. These stimulated cells rapidly proliferate to focal sites of injury due to an increasing expression of immunoreactive cell surface molecules and to the secretion of various inflammatory molecules such as chemotactic factors which induce the recruitment of other microglial cells. Figure 3 shows the changes in microglial morphology and accumulation of activated microglia in a mouse stroke model (48 hours after transient 30 minutes middle cerebral artery occlusion). This phenomenon is accompanied by an increased expression of cytokines: interleukins (IL-1β, IL-4, IL-6, IL-10), tumor necrosis factor-α (TNFα), interferons and chemokines such as MCP-1 [19, 20]. The surrounding astrocytes are sensitive to the increased release of these immunomodulatory peptides and therefore severe ischaemia also compromises astrocytic function. Astrocytes modulate the phagocytic functions of microglia and promote the expression of adhesion molecules in the neurovascular unit on endothelial cells and circulating leukocytes [21, 22]. These early inflammatory processes are likely to be deleterious for neuron survival.

Figure 3
Activation and accumulation of microglia after cerebral ischaemia. Shown are examples of immunohistochemical staining for CD11b, an integrin surface marker on microglial cells, after 48 hours of reperfusion following 30 minutes middle cerebral artery occlusion (MCAo) in mice. Hyper-ramified (A, arrow heads) or activated microglia (A, white arrows) can be detected in the hippocampus (nuclei are stained in blue with DAPI). Phagocytotic microglia are present in the injured brain tissue (B, black arrows). Cresyl violet stained sections of ischemic brains are shown above, red boxes locate the immunolabelled ischaemic tissue in the hippocampus and the striatum shown in A and B. Magnification 20×. Accumulation of activated amoeboid microglia is found in the ischaemic striatum (C), while the contralateral healthy region shows no CD11b positive staining (D). The dashed red circle outlines the ischaemic tissue. Magnification 5×.



Leukocyte infiltration

Shortly after the onset of injury the blood brain barrier opens by the

Figure 4
Therapeutic interventions targeting the inflammatory response after ischaemia. Experimental and clinically tested approaches to reduce brain damage and inflammation after stroke. In red is shown thrombolysis used to dissolve the blood clot [3]. In black, strategies to block excitotoxic pathways, such as the inhibition of glutamate receptors [53] and JNK stress signaling pathway [33, 34]. In blue are shown therapeutic interventions targeting inflammation, such as inhibitors of free radicals [41, 54, 55] and anti-inflammatory molecules [38, 40, 56–58]. And in pink other potential targets such as MMPs [59], PARP-1 [60, 61], inhibitors of caspase-1 or caspase-3 [62, 63] and erythropoietin (EPO) as a neuroprotective agent [64].

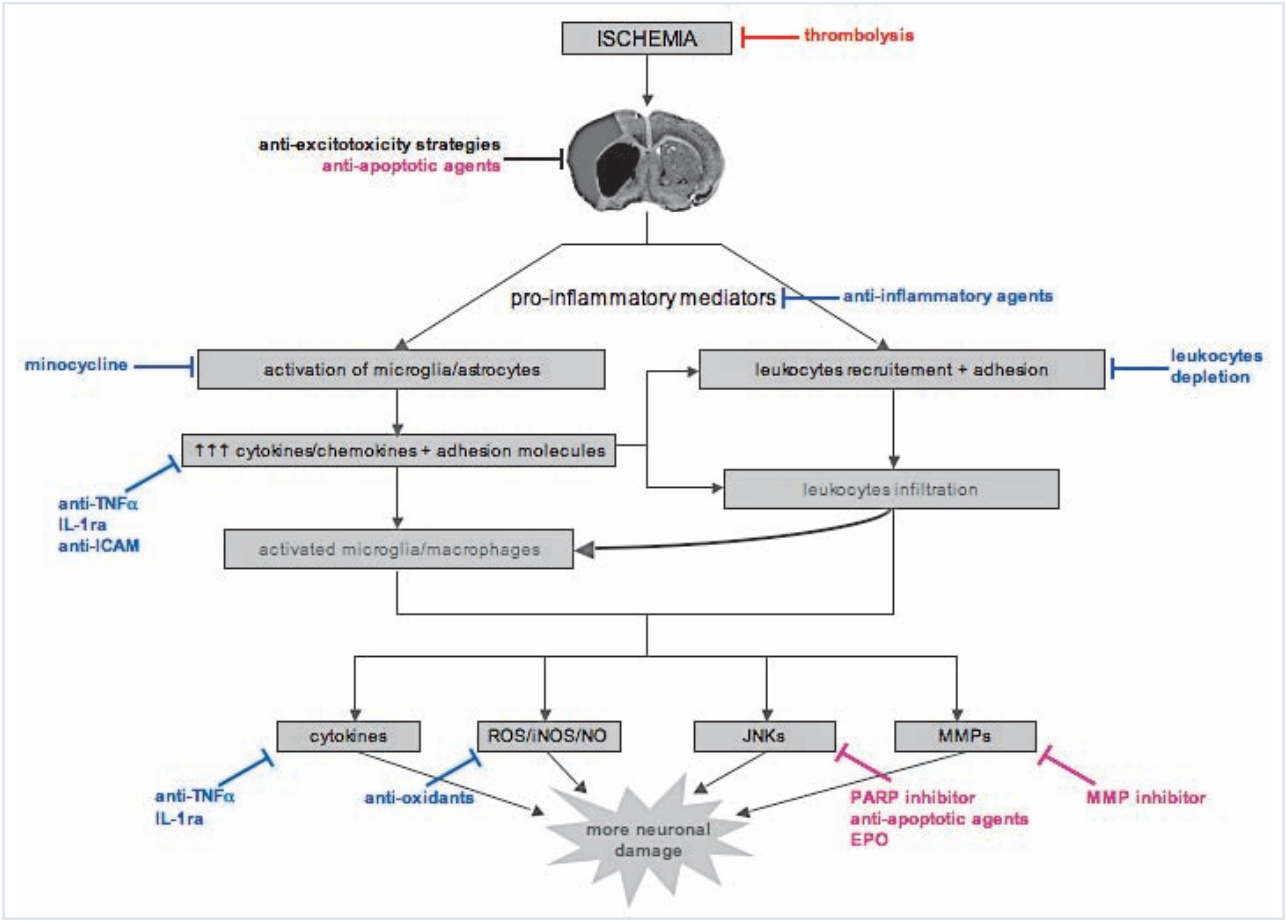


Table 2

	Target	Effects	Reference
Thrombolysis	tPA (alteplase)	Restores blood flow	[3]
Anti-excitotoxicity strategies	NMDA and AMPA antagonists, channel blockers, JNKs inhibitors	Block excitotoxicity pathways	[33, 34, 53]
Anti-oxidants	iNOS and COX inhibitors, NXY-059	Free radical production inhibitors	[41,54,55]
Anti-inflammatory agents	Leukoytes depletion	Reduces the number of circulating neutrophils	[56]
	Anti-ICAM-1 (Enlimomab)	Blocks leukocytes adhesion and transendothelial migration	[40]
	TNF	Prevents TNF from interacting with its receptor	[57]
	Interleukin-1 receptor antagonist (IL-1ra)	Prevents IL-1 from interacting with its receptor	[38]
	Minocycline	Inhibits cytotoxic agents secreted by microglia	[58]
Other	PARP inhibition	Blocks cell death	[60, 61]
	MMP inhibition: MMP-9 knock-out mice	Reduction of proteolytic degradation of BBB	[59]
	Erythropoietin (EPO)	Neuroprotective	[64]
	Caspase inhibitors: Casp-1,-3	Inhibition of apoptosis	[62, 63]

disruption of the endothelia tight junctions [23]. The release of inflammatory mediators from activated glial cells induces the expression of proteins on the outer cell membrane of vascular endothelial cells and leukocytes. The opening of the barrier and the release of inflammatory mediators leads to the migration of circulating leukocytes to the site of injury. Infiltration of leukocytes occurs in three steps: rolling on the surface of endothelial cells, adhesion to the endothelial wall and migration or diapedesis. The initial capture and migration is mediated by three main groups of cell adhesion molecules: selectins (P-, E- and L-selectins), immunoglobulins (VCAM-1, ICAM-1) and integrins (CD11a-c) [11, 24]. Circulating monocytes/macrophages are also recruited at the site of injury and will penetrate into the parenchyma. The proportion of invading macrophages in the ischaemic tissue can not be discriminate from resident activated microglia.

Activation and accumulation of leukocytes (granulocytes, monocytes/macrophages, lymphocytes) at the site of injury results in further damage. Current evidence suggests a detrimental role of inducible or immunological nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) from neutrophils and vascular cells in the ischaemic brain [25, 26]. They induce the formation of reactive oxygen species: nitric oxide (NO) and superoxide respectively, leading to the generation of peroxinitrite [14, 27] which is a powerful oxidant which triggers damage to DNA and other cell constituents [1]. Up-regulation of pro-inflammatory molecules and reactive oxygen species after cerebral ischaemia are not the only cause of secondary injury. Expression of metalloproteinase genes is related to the presence of inflammatory cells in ischemic tissue. A recent study has demonstrated the infiltration of metalloproteinase-9 positive (MMP-9+) neutrophils after human stroke [28]. MMP-9 is involved in the degradation of components of the extracellular matrix and basal lamina which may potentiate haemorrhagic complications after ischaemic stroke.

Transcriptional regulation of inflammation

The secretion of inflammatory molecules in cerebral ischaemia triggers the activation of several transcription factors involved in the inflammatory response. The nuclear factor-kappa B (NF-κB) when activated, induces the expression of genes encoding cell adhesion molecules, cell surface receptors and cytokines [29, 30]. Iadecola and colleagues have shown an attenuation of the inflammatory response induced by focal cerebral ischaemia in mice with a null mutation in the CD36 receptor which recognises pathogens and induces an inflammatory response through the activation of NF-κB [31]. Interference with the nuclear factor-kappa B activation may therefore be beneficial to secondary ischaemic injury.

Mitogen activated protein kinases (MAPKs) are a family of key proteins which are activated in response to stress signals. MAPK signalling pathways positively regulate transcription of inflammatory genes, such as those coding for TNF-α, IL-1β, IL-6, IL-8, COX-2 (for review [9, 32]). D-JNKi-1, a specific inhibitor of the JNK pathway, was shown to induce a strong neuroprotective effect in a range of experimental cerebral ischaemia model [33, 34]. Besides, the inhibition of MAP kinases, especially p38 and JNK, could lead to a reduction in pro-inflammatory molecule production by inflammatory cells, especially microglia/macrophages in which the MAPK cascades are highly activated after an ischaemic injury [35,36].We are therefore currently investigating the effect of D-JNKi-1 on the inflammatory response and the progression of the tissue damage after ischaemia.

Neuroprotective approaches targeting inflammation

Different strategies aimed at preventing the inflammatory response after cerebral ischaemia have been successful in rodent models. Ischaemic damage was shown to be attenuated, together with systemic leukocyte depletion by preventing the expression of, or by blocking adhesion molecules, by inhibiting pro-inflammatory cytokines or by diminishing the free radical generating enzymes iNOS or COX-2 (for review [1, 37]). Attempts to translate therapeutic interventions to stroke patients have been more disappointing than promising. For instance the selective IL-1 receptor antagonist (IL-1ra) which limits the pro-inflammatory action of IL-1, has been tested in randomised patients with acute stroke [38]. Despite a conclusive phase II study no recent publications have reported IL-ra as a therapeutic agent for acute stroke.

More than a thousand of potential agents underwent clinical evaluation [39]. However none of these drugs have demonstrated benefit in stroke clinical trials as for instance the application of murine monoclonal anti-ICAM-1 in Enlimomab trial [40] and XY-059, a nitrone-based free radical trapping agent [41]. A non-exhaustive list of therapeutic strategies targeting especially inflammation after ischaemic stroke is shown in figure 4 and table 2.

Experimental studies to clinical trials: lost in translation

Reasons for failure have been discussed before [42] and include morphological and functional differences between rodents and humans, timing of the intervention, evaluation of efficacy, pharmacokinetic issues (plasma concentration of drugs) and side effects. Anti-inflammatory strategies have also in some cases promoted deleterious infections and fever [40]

Until now, researchers have focused mostly on the negative role of inflammation after stroke and thus have looked to therapeutic means to inhibit post-stroke

inflammation. Nevertheless, there is evidence suggesting that inflammation might also be beneficial in stroke: it is a crucial mechanism to clear damaged tissue after an infarction, it promotes angiogenesis, tissue remodelling and regeneration [1, 43]. Therefore, there is clearly the need to better understand the subtle balance between the beneficial and deleterious effects of inflammation in stroke. Furthermore, experimental studies on cerebral ischaemia have mostly target one cell type, i.e., neurons, while endothelium, astrocytes and microglia have been considerably neglected. Future research on experimental stroke models should considered the important role of non-neuronal cells and the bivalent function of inflammation. A better insight in these aspects is important before planning future clinical trials.

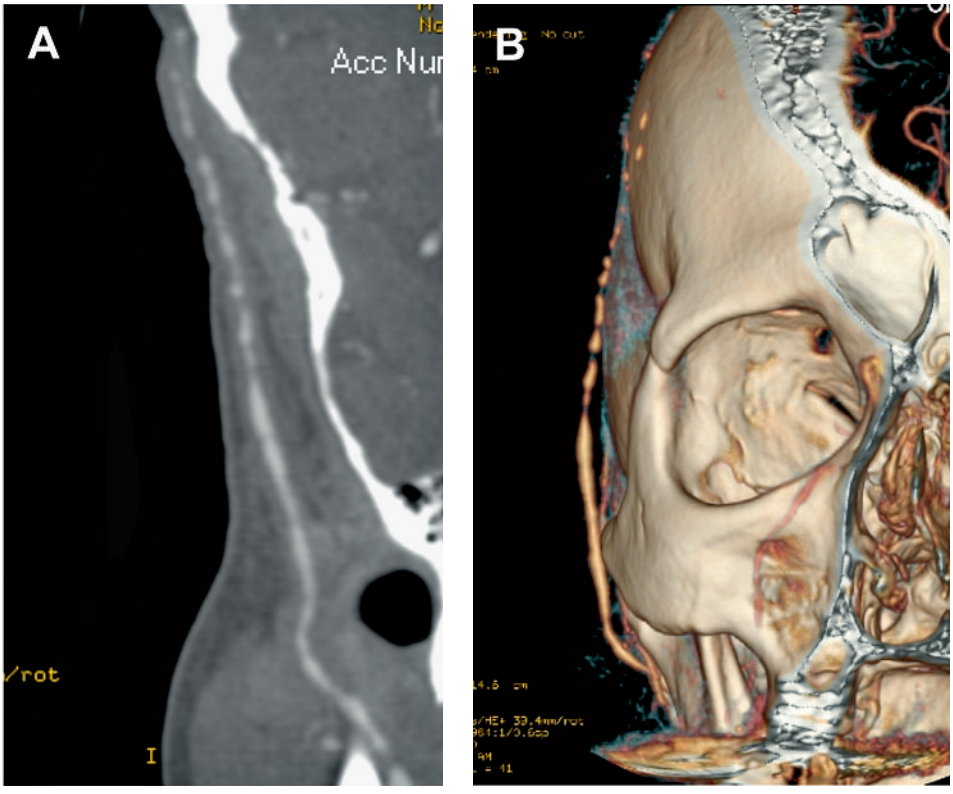
Autoimmune and infectious aetiologies of stroke

We have discussed on the inflammatory response after cerebral ischaemia and its consequences. In this second part of this review we will illustrate how a disorder affecting the immune system could lead to stroke. Therefore, we will briefly review the vasculitis which can cause stroke. Primary vasculitis is classified into three categories, depending on the size of the affected arteries. This classification is relevant for the clinician. Indeed, magnetic resonance angiography or even conventional arteriography can provide good information on large-size arteries, are of variable value in middle-size arteries vasculitis and are useless in small-size arteries vasculitis, since in the latter case, the lesions are below the threshold of detection (tab. 1). In other terms, a normal neuro-imaging study does not rule out a middle-size or a small-size arteries vasculitis. A meningeal and brain parenchymal biopsy might thus be warranted. Another important fact is that most vasculitis that can cause strokes are systemic diseases and thus other organs, including the heart, are frequently involved, for instance the Takayasu arteritis (TA) which affects the large arterial trunks. TA must always be ruled out in young patients with stroke, especially if they are female and of Asian descent. Stroke, either ischaemic or haemorrhagic occurs in 20–30% of TA. Treatment consists in a combination of corticosteroids, immunosuppressive therapies and surgery [44].

By contrast, temporal arteriitis (Horton's disease) affects elderly people (usually >60 years old) and is characterised by headaches and a high erythrocyte sedimentation rate (>50 mm/hour) (fig. 5). When suspecting Horton's disease, one must immediately administer high doses of corticosteroids, since there is a risk of occlusion of the central artery of the retina, leading to definite loss of vision. Stroke may occur in 10% of patients with Horton's disease. Other manifestations include fever, fatigue, jaw claudication and its very frequent association with polymyalgia rheumatica. Of importance, temporal artery biopsy, which is the gold standard diagnostic procedure, remains positive up to 7–10 days after corticosteroids were started. The duration of corticosteroid therapy depends on the erythrocyte sedimentation rate and the clinical symptoms. Usually, the treatment lasts a minimum of one year [45].

Primary angiitis of the central nervous system (PACNS) is a rare condition, but very difficult to diagnose. Indeed, in contrast to other vasculitis, PACNS affects only the vessels of the brain (middle-size arteries), without any other systemic manifestations. Its mode of presentation is variable including, in addition to ischaemic or haemorrhagic strokes, confusion, cognitive deterioration, headaches, etc. Extensive

Figure 5
Giant cell temporal arteriitis (Horton's disease).
A Angiographic CT of the head and neck showing skipped stenosis of the right temporal artery in a patient suffering from Horton's disease.
B Three-dimensional reconstruction precising the anatomy and the lesions of this artery.
(With courtesy of the Division of Neuroradiology, Lausanne, Switzerland.)



blood tests are not contributory. Cerebrospinal fluid examination is abnormal in 50–90% of cases, but of little help, since it shows only aspecific findings, i.e., a mild to moderate increase in proteins, and/or leucocytes, and/or erythrocytes.

Nevertheless, if a PACNS is suspected, it is of importance to establish the diagnosis as the treatment is heavy, consisting in long-term corticosteroids and cyclophosphamide. Therefore, a meningeal and parenchymal brain biopsy is often necessary [46].

We will not address here the other primary vasculitis causing stroke (tab. 1), as strokes are relatively rare in those vasculitis and, usually, other organs are affected before the central nervous system. The same is true for secondary vasculitis. Nevertheless, it is worth to mention here the problem of the anti-phospholipid syndrome (aPLs). Indeed, this condition is found in 40% of the cases of systemic lupus erythematosus, but can also be primary, i.e., not associated with any underlying condition. aPLs may cause arterial and venous thrombosis. Typical manifestations include: spontaneous abortion beyond 10 week of gestation, cardiac valve abnormalities, thrombocytopenia, haemolytic anaemia. A neurological involvement is frequent, consisting in strokes, migraine-like phenomenon, chorea, and transverse myelopathy. The diagnosis of aPLs is based on the Sapporo criteria established in 1999 and revised in 2004 in Sydney. Detailing these diagnostic criteria would be well beyond the purpose of this small review and we advise the avid reader to consult the following references: [47–49].

From a neurological standpoint, it is recommended to rule out an aPLs in patients younger than 45 years-old who present with an ischaemic stroke. But it is crucial to follow the above-mentioned modified Sapporo criteria in order to avoid over-diagnosis of aPLs. Indeed, this diagnosis often implies a long-term anticoagulation treatment [50].

Conclusion

Our knowledge on pathophysiology of cerebral ischaemia has greatly improved because of experimental *in vitro* and *in vivo* studies. A large number of drugs have been developed in the purpose to inhibit the complex cascade taking place after stroke including excitotoxicity and inflammation. Despite those efforts none of the potential therapeutic agent has been successful in clinical trials so far.

In this short review we have shown that inflammation can cause an occlusion of a brain artery and therefore drive to cerebral ischaemia, as well as be the direct consequence of stroke and exacerbate damage. Furthermore, stroke induces immunodepression and favors opportunistic infections such as bronchopneumonia [51].

Therefore, in stroke therapy there is a great need to identify new approaches which could block the detrimental inflammatory response as well as inducing neuroprotection or perhaps in combination with thrombolysis.

Acknowledgments

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JNK inhibition and inflammation after cerebral ischemia ☆

Corinne Benakis [a], Christophe Bonny [b], Lorenz Hirt [a,*]

[a] Department of Neurology, University Hospital (CHUV), Lausanne, Switzerland
[b] Xigen Pharmaceuticals, Lausanne, Switzerland

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ABSTRACT

The c-Jun-N-terminal kinase signaling pathway (JNK) is highly activated during ischemia and plays an important role in apoptosis and inflammation. We have previously demonstrated that D-JNKI1, a specific JNK inhibitor, is strongly neuroprotective in animal models of stroke. We presently evaluated if D-JNKI1 modulates post-ischemic inflammation such as the activation and accumulation of microglial cells.

Outbred CD1 mice were subjected to 45 min middle cerebral artery occlusion (MCAo). D-JNKI1 (0.1 mg/kg) or vehicle (saline) was administered intravenously 3 h after MCAo onset. Lesion size at 48 h was significantly reduced, from 28.2 ± 8.5 mm^3 ($n = 7$) to 13.9 ± 6.2 mm^3 in the treated group ($n = 6$). Activation of the JNK pathway (phosphorylation of c-Jun) was observed in neurons as well as in Isolectin B4 positive microglia. We quantified activated microglia (CD11b) by measuring the average intensity of CD11b labelling (infra-red emission) within the ischemic tissue. No significant difference was found between groups. Cerebral ischemia was modelled *in vitro* by subjecting rat organotypic hippocampal slice cultures to oxygen (5%) and glucose deprivation for 30 min. *In vitro*, D-JNKI1 was found predominantly in NeuN positive neurons of the CA1 region and in few Isolectin B4 positive microglia. Furthermore, 48 h after OGD, microglia were activated whereas resting microglia were found in controls and in D-JNKI1-treated slices.

Our study shows that D-JNKI1 reduces the infarct volume 48 h after transient MCAo and does not act on the activation and accumulation of microglia at this time point. In contrast, *in vitro* data show an indirect effect of D-JNKI1 on the modulation of microglial activation.

1. Introduction

Cerebral ischemia induces neuronal damage in the territory of the occluded artery within minutes of the onset of ischemia. The injured neurons undergo cellular degeneration through excitotoxic mechanisms such as the expression of apoptotic proteins and cytotoxic enzymes as well as the up-regulation of stress-signaling pathways (Dirnagl et al., 1999; Mehta et al., 2007). This emergency state strongly activates nearby glial cells leading to the release of inflammatory mediators within the ischemic tissue and to the expression of adhesion molecules in vascular endothelial cells. The inflammatory response leads to the accumulation and activation of microglial cells as well as the recruitment of circulating T lymphocytes to the infarct area (Petty and Wettstein, 2001; Ishikawa et al., 2004; Wang et al., 2007; Benakis et al., 2009). It is postulated that this local inflammatory response contributes to the secondary injury to potentially viable tissue which could lead to a larger cerebral infarct and impaired clinical outcome in patients with ischemic stroke (Barone and Feuerstein, 1999; del Zoppo et al., 2001).

Despite promising data in experimental research, the inhibition of the delayed inflammatory response has failed to improve the outcome after stroke in clinical trials (Enlimomab Acute Stroke Trial Investigators, 2001; Sughrue et al., 2004). There is a great need to further characterize the cellular and molecular mechanisms underlying inflammation to improve chances of identifying promising therapeutic targets to prevent secondary injury. Furthermore, it is now clear that the efficacy of neuroprotective agents is greater if they target not only one single aspect of the ischemic cascade, but multiple mechanisms such as combined inhibition of neuronal cell death and of the detrimental effects of inflammation after stroke (Kaminska, 2005). Experimental protocols therefore need to explore such combined approaches.

D-JNKI1 is a selective non-ATP-competitive inhibitor that prevents the interaction of JNK with its targets including c-Jun (Bonny et al., 2001). This peptide prevents NMDA-induced neuronal death in primary cultures (Borsello et al., 2003; Centeno et al., 2007) as well as rescues the evoked potential response recorded in the CA1 region after oxygen and glucose deprivation (OGD) in organotypic hippocampal slice cultures (Hirt et al., 2004). Moreover, D-JNKI1 remarkably attenuates the lesion size in transient and permanent models of cerebral ischemia in both rats and mice (Borsello

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* Corresponding author. Address: Laboratory of Neurology, CHUV BH 19-208, 1011 Lausanne, Switzerland. Fax: +41 21 3141244.
E-mail address: Lorenz.Hirt@chuv.ch (L. Hirt).

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et al., 2003; Hirt et al., 2004; Repici et al., 2007; Esneault et al., 2008), even with delayed intravenous administration (Wiegler et al., 2008). These experimental studies have also shown that D-JNKI1 improves functional outcome at later time-points after ischemia. All together, these data strongly suggest that inhibition of the JNK pathway is a very promising target for stroke patients.

The JNK pathway is also activated in microglial cell cultures after stimulation by LPS or thrombin (Hidding et al., 2002; Waetzig et al., 2005), which leads to the expression of inflammatory molecules such as TNFα. It is well described that microglia undergo morphological changes after cerebral ischemia with accumulation and proliferation at the site of injury and secretion of inflammatory modulators, such as cytokines, chemokines and toxic factors (Feuerstein et al., 1998; del Zoppo et al., 2000).

Our aim was to test whether D-JNKI1 was also inhibiting the JNK pathway in microglia and if this contributed to the strong neuroprotective effect of delayed D-JNKI1 administration in our models. We examined whether the activation of microglia was reduced in D-JNKI1-treated animals, subjected to middle cerebral artery occlusion (MCAo), by investigating morphological changes and accumulation within the infarct area. Our data show that D-JNKI1 does not influence global morphology and number of microglia *in vivo*. We showed that D-JNKI1 is present mainly in neurons and in few microglia when administered in an *in vitro* ischemia model by subjecting hippocampal slice cultures to OGD. Interestingly, we identified changes in microglia *in vitro*, such as a modification of microglial morphology and localization after OGD or OGD + D-JNKI1. While D-JNKI1 has a strong neuroprotective effect in our stroke model, it does not appear to attenuate the activation by morphological criteria and accumulation of microglia in ischemic tissue at 48 h.

2. Materials and methods

2.1. Animals

Male Crl:CD1/ICR mice (20–30 g, 4- to 5-week-old) and gestant Sprague–Dawley (OFA) rats were purchased from Charles River Laboratories Inc. (Charles River France, L'Abresle, France). All procedures were in accordance with the Swiss Federal Law on Animal Welfare and were approved by the Swiss Federal Veterinary Office.

2.2. Transient middle cerebral artery occlusion (MCAo)

Focal cerebral ischemia was induced as previously described (Wiegler et al., 2008), but with a different anaesthesia protocol and a longer ischemia duration. CD1 mice were anesthetized and maintained with 1–3% isoflurane in 70% N_2O and 30% O_2 using a face mask. Body temperature was maintained at 37 ± 0.5 °C throughout surgery (FHC Inc., Bowdoinham, ME, US). Regional cerebral blood flow (CBF) was continuously recorded by laser Doppler flowmetry (LDF, Periflux 5000, Perimed, Sweden) during a period covering the induction of ischemia until 10 min after the end of ischemia. Transient left MCAo was induced as previously described (Lei et al., 2009) with a silicone-coated nylon monofilament (diameter: 0.17 mm, Doccol Co., Redlands, CA, USA) inserted through the common carotid artery and withdrawn after 45 min to allow reperfusion. Mice were given 0.025 mg/kg of buprenorphine subcutaneously for post-surgery analgesia and were housed in an incubator at 31 °C for 24 h for recovery.

2.3. Groups, treatments and neurological deficits

Randomly, saline solution (NaCl 0.85% Medium, bioMérieux) or D-JNKI1 (0.1 mg/kg, Xigen Pharmaceuticals) was injected intravenously (i.v.) into the tail vein of mice using a 1 ml syringe (Omnifix-1 ml, Terumo needle 25G) and a mouse restrainer (Braintree Scientific Inc.) 3 h after ischemia onset. The lesion volume was evaluated 48 h after cerebral ischemia and immunostainings were performed at 9 h, 24 h, 48 h and 7 days after surgery. The neurological deficit was assessed at 48 h. Neuroscore was graded for severity using the following scale: 0 = no observable neurological deficit, 1 = failure to extend the right forepaw, 1.5 = intermittent circling behavior, 2 = circling behavior, 3 = loss of walking or uncontrolled movement (Bederson et al., 1986; Hirt et al., 2004).

2.4. Tissue preparation and immunohistochemistry

Mice were anesthetized with intra-peritoneal injection of 8 mg/kg xylazine (Rompun® 2%, Bayer) + 100 mg/kg ketamine (Ketanarkon 100, Streuli Pharma AG) and transcardially perfused with PBS followed by 4% paraformaldehyde. Serial coronal 20-μm thick, 720-μm distant cryostat sections were used for lesion size measurements and immunolabelling.

2.4.1. Histological evaluation of neuronal damage

Digitalized images of the Nissl stained tissue were acquired under a light stereomicroscope (Leica MZ16FA) and the ischemic area was measured by a blinded observer with ImageJ 1.38x software (NIH, http://rsb.info.nih.gov/ij/). The direct infarct volume was calculated by multiplying the sum of the infarct areas on each section by the distance between the sections.

2.4.2. Staining of degenerating neurons: FluoroJade B

FluoroJade B staining (0.0004% FluoroJade® B, Millipore, UK) was performed on frozen sections according to the Chemicon International Company protocol. Sections were then mounted in Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

2.4.3. Immunohistochemistry staining for glial and inflammatory cells

Sections were blocked in 0.3% H_2O_2 for 20 min at room temperature (RT) followed by 10% normal goat serum (NGS, Invitrogen, Paisley, UK) in PBS with 1% bovine serum albumin (BSA) for 1 h at RT. Sections were incubated overnight at 4 °C with the following primary antibodies: rat anti-CD11b (Mac-1, 1:100, AbD SEROTEC) or biotinylated Isolectin B4 (1:500, *Griffonia simplicifolia* lectin I, Vector Laboratories) to label microglia, mouse anti-GFAP (1:500, Millipore) for astrocytes, hamster anti-TCRbeta (1:200, BioLegend) for T lymphocytes, hamster anti-CD11c (1:200, BioLegend) for dendritic cells. Using the immunoperoxidase method, biotinylated anti-rat (1:500, BA-9400, Vector Laboratories, Burlingame, CA), anti-mouse (1:500, Vector Laboratories, Burlingame, CA) or anti-hamster (1:500, Biolegend) IgG antibodies were applied for 1 h followed by avidin–biotin–horseradish peroxidase complex (Vectastain kit, Vector Laboratories, Burlingame, CA). Incubation with biotinylated secondary antibody was omitted for Isolectin B4. The immunocomplex was visualized by 3,3′-diaminobenzidine (DAB kit, Vector Laboratories, Burlingame, CA) and mounted with Eukitt.

2.4.4. Double immunofluorescence

After blocking in 10% NGS (±0.1% Triton X-100) sections were incubated overnight at 4 °C in various combinations of the following primary antibodies: mouse anti-NeuN (1:500, Millipore), mouse anti-GFAP (1:500), biotinylated Isolectin-B4 (1:500) and rabbit anti-phospho-c-Jun(Ser63) or phospho-c-Jun(Ser73) (1:100, Cell Signaling). Antigens were visualized with the appropriate fluorochrome-conjugated secondary antibodies: alexa fluor 488 anti-rabbit (1:500, Invitrogen), alexa fluor 594 anti-mouse (1:500, Invitrogen), Cy3-conjugated Streptavidin (1:500, Jackson ImmunoResearch Laboratories) for 1 h at RT. Sections were counterstained with DAPI (1:10,000, 1 mg/ml, SIGMA) and mounted in

Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). Images were acquired with the Aviovision v3.1 software in a Zeiss Axiovision microscope. No specific immunoreactivity was observed in control sections prepared by omitting primary antibodies.

2.5. Quantification of CD11b positive cells

Rat CD11b antigen was revealed using the secondary antibody IRDye 800-labeled anti-rat IgG (1:2000, Rockland) for 1 h at RT. CD11b infra-red emission was measured at 800 nm with an Odyssey infrared imaging scanner (Li-COR, Biosciences, Lincoln, Nebraska, USA). The sections were counterstained with Nissl after the first scan to avoid detection of Nissl blue staining in the 800 nm channel, and rescanned at 700 nm for delimitation of the ischemic zones. The average intensity of microglia ($AvIntCD11b_{Total}$) was calculated within the ischemic region for each brain as follows: $AvIntCD11b_{Total} = (\Sigma_i RawIntensity/\Sigma_i pixels) - AvInt_{Background}$, with $i = i$th coronal section of a brain, $\Sigma_i RawIntensity/\Sigma_i pixels$ = AvIntCD11b without background correction and $AvInt_{Background}$ = average intensity of the background measured by omitting the primary antibody. One animal was excluded in the vehicle-treated group, because of unconvincing IRD-800 labelling.

2.6. Organotypic hippocampal slice cultures (OHC)

Three hundred and fifty micrometers thick coronal hippocampal slices from 10- to 12-day-old Sprague–Dawley rats were cultured on porous membrane units (Millicell-CM, Millipore, UK) in wells containing 1 ml of culture medium with 25% horse serum (Oxoid, UK), 50% minimal essential medium supplemented with HEPES and sodium bicarbonate (MEM, Gibco, UK), 25% Hanks' balanced salt solution (HBSS, Gibco, UK), 2 mM L-glutamine and 36 mM D-glucose. Cultures were grown at 33 °C, 100% humidity and 5% CO_2. After 3 days the medium was replaced by fresh culture medium and, after 6 days, by a medium with 15% horse serum, 60% MEM, 25% HBSS, 2 mM L-glutamine and no glucose (Badaut et al., 2005).

2.7. Oxygen and glucose deprivation (OGD) and quantification of cell death

Hippocampal slice cultures were subjected to *in vitro* ischemia by exposure to reduced oxygen and glucose concentrations as described previously (Hirt et al., 2004). At day 8, slices were transferred to a serum-free hypoglycemic medium, DMEM (D5030; Sigma, USA) supplemented with 1 mM D-glucose and 2 mM L-glutamine, in a humidified hypoxia chamber (COY, USA) with a hypoxic atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 37 °C for 30 min. Control cultures were placed at 37 °C for 30 min, in a humid normoxic atmosphere. D-JNKI1 diluted in PBS (final concentration 12 nM) or an equal volume of PBS was added 6 h after OGD. Cell death was determined in the CA1 region using the fluorescent viability indicator propidium iodide (PI, final concentration 5 μg/ml, Sigma). PI fluorescence emission (excitation wavelength 568 nm) was measured 48 h after OGD and quantified densitometrically, using ImageJ software. After subtracting the background fluorescence, the results were expressed as a percentage of maximal cell death obtained by incubating the hippocampal slice cultures in PBS for 24 h at 4 °C. Cell death was averaged for the four slices of each culture.

2.8. Slice preparation and immunofluorescence

OHC were fixed in 4% paraformaldehyde for 1 h at 4 °C, blocked 1 h at RT in 10% NGS and incubated overnight at 4 °C with the following primary antibodies: biotinylated Isolectin-B4 (1:500), mouse anti-NeuN (1:500), rabbit anti-phospho-c-Jun(Ser63) or phospho-c-Jun(Ser73) (1:100) and rabbit anti-D-JNKI1 (1:300, Xigen Pharmaceuticals, Switzerland). After rinsing in PBS, slices were incubated with Cy3-conjugated Streptavidin (1:5000), alexa fluor 594 anti-mouse (1:200) or alexa fluor 488 anti-rabbit (1:200) for 1 h at RT, mounted using Vectashield Mounting Medium and viewed under a Leica SP5 confocal laser-scanning microscope.

2.9. Statistical analysis

Results are expressed as mean ± SEM, except for neurological score which is expressed as [median, min, max]. Data were analyzed using two-sample *t*-test (GraphPad Prism software 5.0). Analyses of correlation were done using the SAS software package. Normalization test was performed to check for significant violations in the distribution assumptions. *P* values <0.05 were considered significant.

3. Results

3.1. Transient MCAo induces an accumulation of inflammatory cells in the ischemic tissue at 48 h

In order to evaluate the effect of D-JNKI1 on inflammation, we investigated the activation and accumulation of glial and inflammatory cells after focal cerebral ischemia in mice by immunohistochemistry. We first characterized microglial activation at different time-points after ischemia onset (9 h, 24 h, 48 h and 7 days). In our MCAo model, we found a peak of CD11b positive microglial cells in the ischemic tissue at 48 h (Supplementary Fig. 1 online). At earlier time-points, there was either no detectable CD11b-staining (9 h), or microglia were in a ramified state with a less dense staining (24 h). At 48 h, there were numerous microglia in the infarct area with an ameboid morphology. Seven days after MCAo, highly ramified microglia were present only in the injured hippocampal tissue, whereas no positive staining was seen in the striatal ischemic region. Further experiments with microglia were performed 48 h after surgery when CD11b staining was most prominent.

At 48 h after 45 min transient MCAo, infarct areas were localized in the striatal and cortical regions of the left hemisphere (Fig. 1A, left) highlighted by a specific marker for degenerating neurons (FluoroJade B, Fig. 1B). The cellular changes resulting in reactive astrocytes are an important component of glial activation after ischemia. It has been previously described that the function of reactive astrocytes is to create a physical barrier between damaged and healthy cells (Anderson et al., 2003). In our model of MCAo, we found that glial fibrillary astrocytic protein positive cells (GFAP+) with a stellate morphology were restricted to the infarct border, outside the ischemic zone, while hypertrophic GFAP+ cells were found within the ischemic tissue, suggesting a reactive gliosis 48 h after MCAo (Fig. 1C). Activated microglia, characterized by an increase in size of the cell body and a decrease of ramifications, were highly present in the ischemic tissue as shown by Isolectin B4 and CD11b positive staining (Fig. 1D and E). It has been postulated that microglia could differentiate into dendritic cells (DCs) and have a phagocytic function (Reichmann et al., 2002). Staining for DCs using CD11c was found in the ischemic region (Fig. 1F). Activation of microglia, dendritic cells and restriction of the extent of damaged tissue by astrocytes after stroke as well as the breakdown of the blood brain barrier could promote the infiltration and accumulation of T lymphocytes within the infarcted area (Raivich et al., 1996; Ishikawa et al., 2004). We performed immunohistochemistry using antibody against the beta chain of a T cell receptor (TCRβ) and found positively stained T lymphocytes especially in the ischemic striatum (Fig. 1G1).

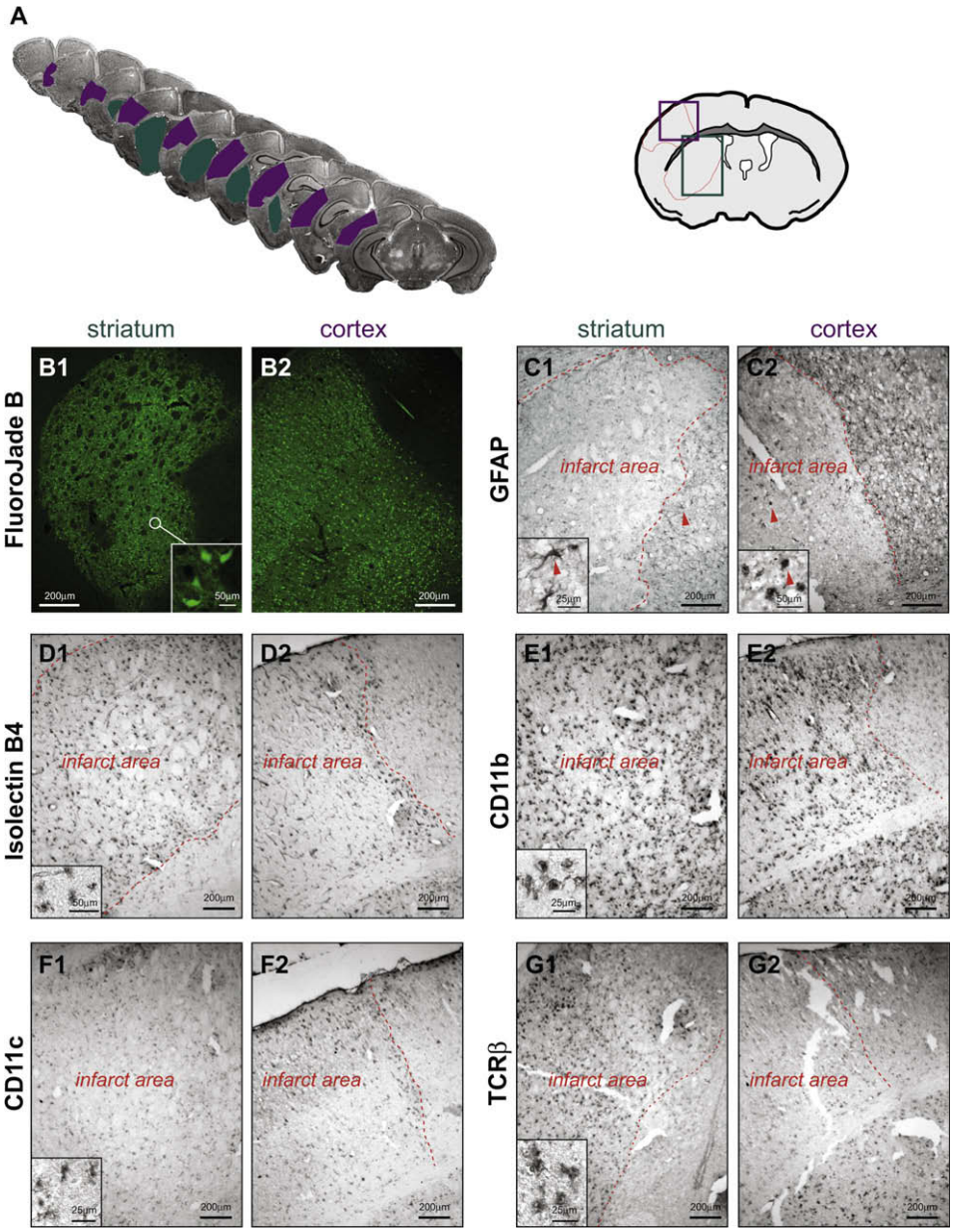

Fig. 1. Glial and inflammatory cells in the ischemic tissue. CD1 mice were subjected to 45 min MCAo and brain sections immunostained 48 h after surgery. (A) Representative Nissl-stained 720-μm distant coronal sections of the total infarct area 48 h after MCAo (left). Striatal and cortical damaged regions of the ipsilateral hemisphere are represented in blue-green and purple, respectively. The location of striatal (blue-green square) and cortical (purple square) ischemic areas where all immunohistochemistry data were analyzed are shown in the upper right panel. (B) Representative histological section 48 h after MCAo showing degenerating neurons (FluoroJade B) located within striatal (B1) and cortical (B2) ischemic lesions in the territory of the left MCA. (C) Reactive astrocytes (GFAP positive cells) are predominantly located in the spared parenchyma bordering the ischemic zone (C1, arrow head), whereas hypertrophic astrocytes are within the demarcated area (C2, arrow head). (D and E) A robust inflammatory response shown by activated (Isolectin B4 and CD11b positive) microglial cells is restricted to the infarct area clearly separated from immediately adjacent viable neural tissue. Inserts show high magnification of typical activated microglia. (F and G) Dendritic cells (CD11c) and T lymphocytes (TCRβ), especially in the striatum (G1) are also detected within the ischemic area and not in the healthy contralateral parenchyma.

Our data show marked inflammatory changes 48 h after transient MCAo with a high degree of activation of glial cells, with reactive astrocytes surrounding the lesion and an accumulation of microglia within the ischemic tissue as well as the presence of inflammatory cells such as DCs and T cells.

3.2. MCAo induces c-Jun phosphorylation in neurons and glial cells

Activation of the JNK pathway after ischemia is well described, resulting in the phosphorylation of JNK targets, including transcription factors in the nucleus such as the immediate early gene *c-jun* (Borsello and Bonny, 2004; Gao et al., 2005; Repici et al., 2007). Using specific antibodies directed against the phosphorylated Serine-63 or -73 residues of c-Jun, we found phospho-c-Jun(Ser73) in cortical region as early as 9 h after MCAo (Supplementary Fig. 2 online) even though we could not detect marked tissue damage, by Nissl staining (see Supplementary Fig. 1). When the lesion was formed, activation of c-Jun was detected essentially in the cortical infarcted areas 24 h after MCAo, while at 48 h positive staining was in both cortical and striatal regions. As late as 7 days after MCAo induction we still observed p-c-Jun labelling in the ischemic hemisphere. The same time course was observed using the specific anti-phospho-c-Jun(Ser63) antibody (data not shown).

In order to further characterize the role of the JNK inhibitor D-JNKI1 not only in neurons but also in glial cells, we performed double-labelling of p-c-Jun(Ser63) using different cell-specific markers as NeuN (neurons), Isolectin B4 (microglia) and GFAP (astrocytes) within the ischemic tissue 48 h after MCAo. Despite early activation of c-Jun at 9 and 24 h, we chose to analyze a possible effect of D-JNKI1 at 48 h after ischemia onset, because we detected a peak of microglial activation (see above) and c-Jun is activated in the entire ischemic tissue at this time point (see Supplementary Figs. 1 and 2). p-c-Jun(Ser63) (green) was detected as expected in the nucleus of apparently non-degenerating neurons, located predominantly in the cortex (red preserved shape, Fig. 2A1, arrow heads), while in the striatal ischemic region we found dying neurons and rare co-localization with p-c-Jun (Fig. 2A2, arrow). We also found a high degree of co-localization of microglia with p-c-Jun (Fig. 2B1–B2, arrow heads). Higher magnification shows nuclear localization of p-c-Jun in microglial cells in an activated ameboid-phagocytic macrophage-like morphology. p-c-Jun was also detected in GFAP+ cells in the cortical and striatal ischemic areas (Fig. 2C, arrow heads).

Taken together, these data demonstrate that c-Jun phosphorylation occurs in the ischemic areas (Fig. 2, boxes on the coronal brain section in the upper left) and is detected in neurons as well as in glial cells 48 h after MCAo.

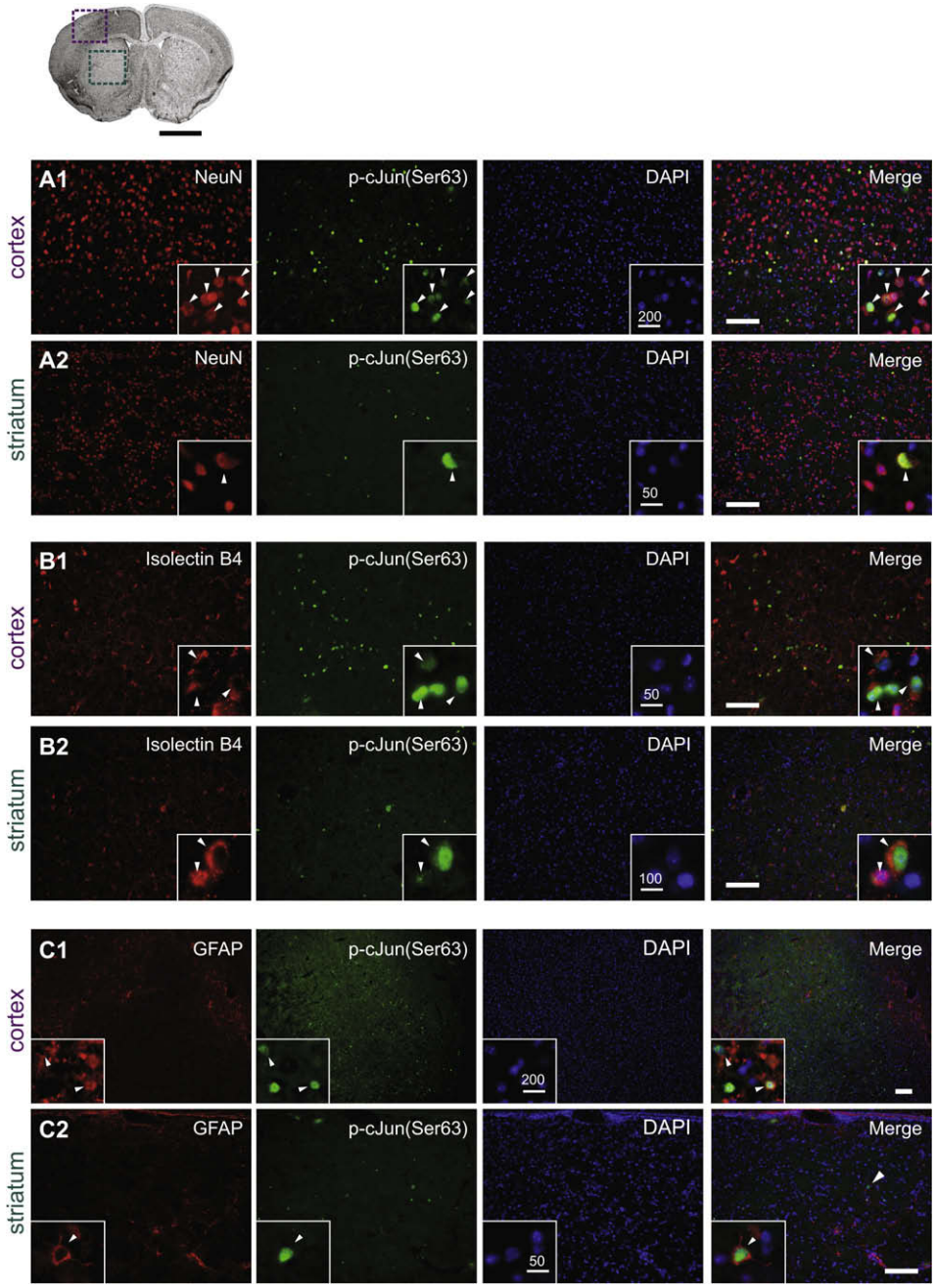

Fig. 2. Activation of c-Jun in neurons and glial cells. The boxes on the brain section in the upper left show the striatal (blue-green) and cortical (purple) ischemic areas where immunofluorescence experiments were carried out. (A–C) MCAo promotes nuclear phosphorylation of c-Jun(Ser63) (p-c-Jun) (lines A1–C1). (A and B) Double fluorescent immunolabelling experiments show that p-c-Jun was highly detected in neurons (NeuN) with an apparently preserved, non-degenerating shape (A1 and A2, arrow heads) as well as in activated microglia (Isolectin B4, B1 and B2, arrow heads). (C) Cytoplasmic GFAP positive cells merged with p-c-Jun are found in the striatal and cortical ischemic areas (C1 and C2, arrow heads). Inserts show high magnification of double labelling. Scale bar: 100 μm.

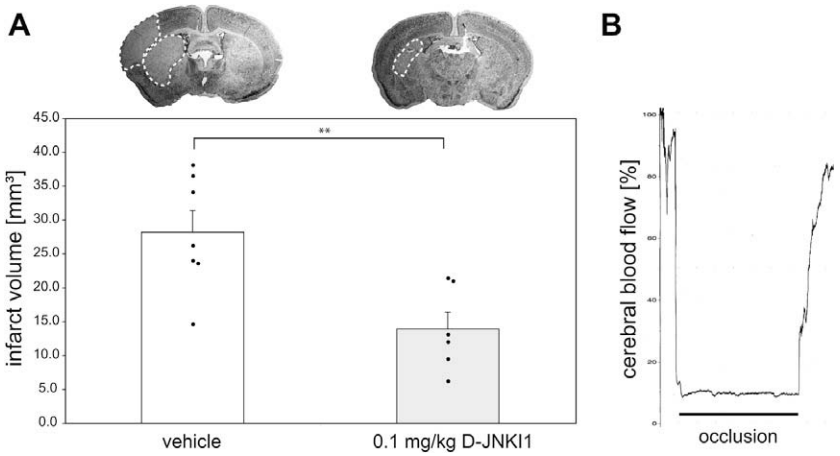

Fig. 3. Neuroprotection with D-JNKI1 3 h after transient MCAo. (A) Intravenous administration of 0.1 mg/kg D-JNKI1 3 h after 45 min MCAo highly significantly reduced the infarct volume compared to vehicle injected controls. Nissl-stained coronal sections show typical examples of lesion ($p = 0.0057$; $^{**}p < 0.01$), circles represent individual animals. (B) Representative diagram of the cerebral blood flow (CBF) as a function of time. The CBF was monitored from the beginning of the surgery until 10 min after withdrawal of the filament to follow the reperfusion.

3.3. The highly neuroprotective JNK inhibitor D-JNKI1 does not reduce the accumulation of microglia in the ischemic tissue 48 h after MCAo

To analyze whether D-JNKI1 has an inhibitory function on the activation and accumulation of microglia at 48 h, we quantified microglial cells after MCAo in treated and non-treated groups (vehicle or D-JNKI1). First, mice were subjected to 45 min MCAo and treated i.v. with D-JNKI1 or vehicle 3 h after MCAo onset. 48 h after surgery, the lesion area could be clearly outlined by Nissl staining (Fig. 3A, dashed line in microphotographs above the diagrams). The direct lesion volume was 28.2 ± 8.5 mm^3 in saline-injected mice ($n = 7$), and 13.9 ± 6.2 mm^3 in the D-JNKI1-treated mice ($n = 6$), providing a significant 51% reduction of the lesion size ($p = 0.0057$), confirming our previous findings (Wiegler et al., 2008), (Fig. 3A). Fig. 3B illustrates a typical Doppler curve measuring the cerebral blood flow from the beginning of the surgery until 10 min after reperfusion. Regional cerebral blood flow was measured by laser Doppler in all animals and is shown in Table 1. Data indicate satisfactory reduction in laser Doppler flow during ischemia in all animals and satisfactory reperfusion, slightly better in the D-JNKI1-treated group ($p = 0.033$). This minor difference in level of reperfusion does not explain the important reduction in lesion volume. Table 1 also shows similar weight and temperature measurements during surgery in both groups. Temperature at sacrifice was lower in vehicle-treated animals than in D-JNKI1-treated mice ($p = 0.048$), which correlated with the reduction of the infarct volume in the D-JNKI1-treated group. Surprisingly, there was no difference in weight between groups before and 48 h after the operation, probably due to the high distribution level of the lesion size in this MCAo model. Neuroscore scaled from 0 (best functional outcome, no deficit) to 3 (worst outcome) was not significantly better in the D-JNKI1-treated group (1.0, 0, 2.0) than in vehicle-treated group (1.0, 1.0, 2.0); $p = 0.701$, most likely due to the lack of sensitivity of this test. We have shown previously a beneficial effect of D-JNKI1 on functional outcome after MCAo using more sensitive tests (Borsello et al., 2003; Hirt et al., 2004; Wiegler et al., 2008).

Table 1
The table shows measurements of cerebral blood flow, weight and temperature in the group treated with vehicle or D-JNKI1. There was a slightly better reperfusion in the D-JNKI1 treated group ($p = 0.0331$; $^{*}p < 0.05$). Temperature at sacrifice was lower in vehicle- than in D-JNKI1-treated mice ($p = 0.0477$; $^{*}p < 0.05$).

	Vehicle ($n = 7$)	D-JNKI1 ($n = 6$)
CBF during ischemia (%)	15.4 ± 2.1	13.2 ± 1.5
CBF after ischemia (%)	50.7 ± 2.3	63.0 ± 4.9*
Weight (g)	25.1 ± 0.9	25.0 ± 0.7
Weight at sacrifice (g)	19.6 ± 1.7	18.5 ± 1.0
T during ischemia (°C)	37.0 ± 0.1	36.7 ± 0.1
T at sacrifice (°C)	35.4 ± 0.2	36.3 ± 0.3*
Neuroscore at 48 h	1.0, 1.0, 2.0	1.0, 0, 2.0

In order to investigate the effect of D-JNKI1 treatment on the activation of microglia, we performed immunohistochemistry using a CD11b-specific antibody in sections from all animals 48 h after surgery. In a blinded study we could not identify morphologic changes (activated-ameboid microglia versus resting-ramified cells) between animals and groups (data not shown, representative CD11b immunostainings of one animal per group are shown in Fig. 4A), suggesting that D-JNKI1 does not influence changes in microglial shape in our MCAo model. We then quantified the number of microglial cells by measuring the average intensity of CD11b (Fig. 4B, green) in the total ischemic area (Fig. 4B, red, dashed line) in untreated mice ($n = 6$) and D-JNKI1-treated mice ($n = 6$) 48 h after MCAo. Quantification analysis of CD11b average intensity did not show a significant difference between the vehicle-treated and D-JNKI1-treated group (Fig. 4C), suggesting that D-JNKI1 does not prevent the accumulation of microglia in the ischemic tissue. Fig. 4D shows the correlation between the expression of CD11b and the brain lesion volume in both groups. As no modification of microglial activation was detectable after D-JNKI1 treatment, we hypothesized that there was a direct link between the activation of microglia and the size of the lesion independently of the treatment. There was a strong linear correlation between CD11b and lesion volume in the vehicle-treated group (black circle, $R = 0.94$, $p = 0.005$). In the D-JNKI1 treated group, this correlation was negative but was not significant (black empty circle, $R = -0.01$, $p = 0.9$). Linear regression analysis between groups showed that there was no significant difference ($R = -0.01$, $p = 0.9$).

These data show that in our stroke model, D-JNKI1 strongly reduces the infarct volume but has no effect on the morphology and accumulation of microglia within the damaged tissue 48 h after MCAo. This suggests that neuroprotection with D-JNKI1 could occur independently of microglial activation in our cerebral ischemia model.

3.4. D-JNKI1 is present mainly in neurons and in some microglial cells 48 h after OGD

The finding that MCAo activates p-c-Jun in microglia and that D-JNKI1 fails to inhibit the activation and accumulation of microg-

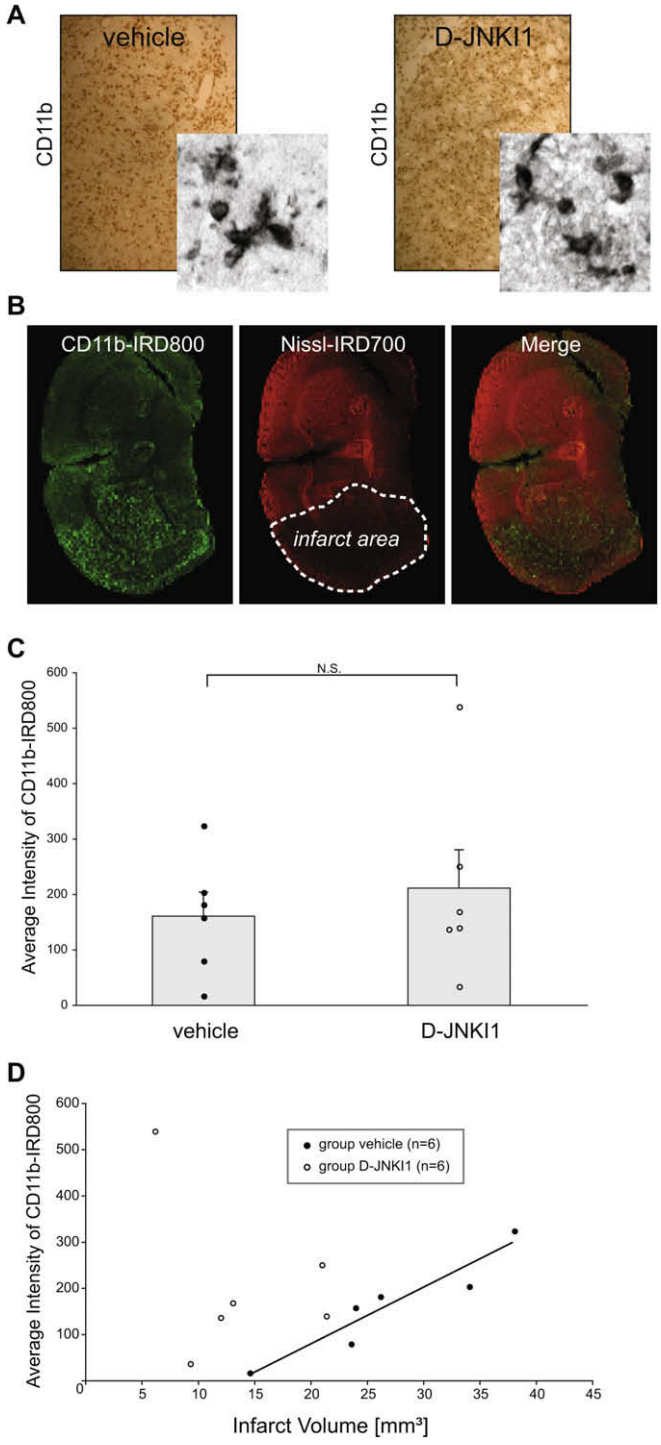

Fig. 4. Quantification of microglia in the ischemic region in treated and untreated groups. (A) Representative CD11b immunohistochemistry of vehicle- and D-JNKI1-treated animals. Inserts show high magnification of CD11b+ microglia. (B) Representative CD11b infra-red emission staining (green) and Nissl-stained section (red) 48 h after MCAo. The measurements were done in the outlined ischemic area (dashed line). Merged scanned microphotograph shows that CD11b staining is localized within the ischemic tissue. (C) Quantification of CD11b average intensity in vehicle- ($n = 6$) and D-JNKI1-treated ($n = 6$) animals in the ischemic tissue. (D) Correlation between infarct volume and the average intensity of CD11b in treated (empty black circle) and untreated groups (black circle). There is a correlation between the infarct volume and the average intensity in vehicle-treated group ($R = 0.94$, $p = 0.005$; $^{**}p < 0.01$). This correlation is negative and not significant in D-JNKI1-treated group ($R = -0.36$, NS). Linear regression analysis of slopes of treated and not treated groups gives a non-significant difference between groups ($R = -0.01$, $p = 0.9$, NS). NS, not significant.

lia despite a strong neuroprotective effect prompted us to investigate the localization of D-JNKI1. The peptide was not detectable in the brain after i.v. injection. Co-localization experiments were therefore assessed in our *in vitro* model of cerebral ischemia as depicted schematically in Fig. 5A. First, we exposed organotypic hippocampal slice cultures to OGD for 30 min and treated cultures 6 h after OGD with 12 nmol/l of D-JNKI1. We measured PI uptake as an indicator of cellular death in the CA1 region of hippocampal slices in control conditions (data not shown), 48 h after OGD and after OGD followed by D-JNKI1 treatment. Fig. 5 shows that treating slices with the neuroprotective peptide significantly reduced the PI uptake from 35.3 ± 8.0% in non-treated slices ($n = 4$) to 4.7 ± 2.9% in D-JNKI1-treated slices ($n = 4$), indicating a significant 87% reduction of neuronal death ($p = 0.012$) confirming previous findings (Hirt et al., 2004).

We then investigated the cellular localization of D-JNKI1 in these slices. We performed double-labelling 48 h after OGD on treated slices using antibodies against D-JNKI1 (green) and neurons (NeuN, red) or microglia (Isolectin B4, red) (Fig. 5C). D-JNKI1 was found in the neuronal CA1 region with a cytoplasmic staining (squares), and a few positive cells were localized outside the CA1 neuronal layer (circles). Co-localization of D-JNKI1 was observed mainly in neurons of the CA1 (Fig. 5C1, arrow heads) and in few microglia outside or at the border of the CA1 region (Fig. 5C2, arrow heads).

Taken together, these observations show that D-JNKI1 is localized in the cytoplasm of CA1 neurons where it is likely to inhibit the JNK death signaling pathway, and that some co-localization of the peptide is also observed in microglia, suggesting that D-JNKI1 could modulate the JNK pathway also in these cells.

3.5. D-JNKI1 modulates the activation of microglia after OGD

We have demonstrated that D-JNKI1 was neuroprotective *in vitro* and was found mainly in neurons as well as some microglia. To verify the peptide's molecular function in these cells, we examined whether D-JNKI1 could inhibit c-Jun phosphorylation and modulate the activation of microglia.

First by Nissl staining we observed that the CA1 layer is well preserved in control slices (Fig. 6A1) and after D-JNKI1 treatment in slices subjected to OGD (Fig. 6A3), whereas neurons after OGD without treatment show the typical shape of dying cells with their pyknotic nuclei (Fig. 6A2). The same results were obtained using the nuclear specific antibody NeuN (Fig. 6B). Fluorescent double immunolabelling using NeuN and p-c-Jun(Ser63) shows, as expected, that in control slices there was no p-c-Jun staining in the neuronal layer (Fig. 6B1). After OGD, the level of p-c-Jun clearly increased in the nuclei of neurons (Fig. 6B2), whereas in treated slices fewer positive p-c-Jun were observed (Fig. 6B3). The same results were obtained using an antibody against p-c-Jun(Ser73) (data not shown). Surprisingly, we observed no colocalisation of p-c-Jun(Ser73) in microglia after OGD and in D-JNKI1-treated slices subjected to OGD (Fig. 6C2–C3).

However, when we analyzed qualitatively microglial morphology *in vitro*, in control slices, we observed microglia with long thin processes, the typical structure of so-called resting microglia, homogeneously localized within the slice (Fig. 6D1), while slices subjected to OGD showed microglia with less ramified processes and a round cell body (ameboid microglia) concentrated within the neuronal CA1 region (Fig. 6D2). In Fig. 6D3, the CA1 layer of D-JNKI1-treated slices shows ameboid-like microglia and more ramified cells localized at the border and within the CA1 neuronal layer, respectively. Therefore, these observations suggest that treatment with D-JNKI1 after OGD modifies microglial activation and localization within the CA1 neuronal layer.

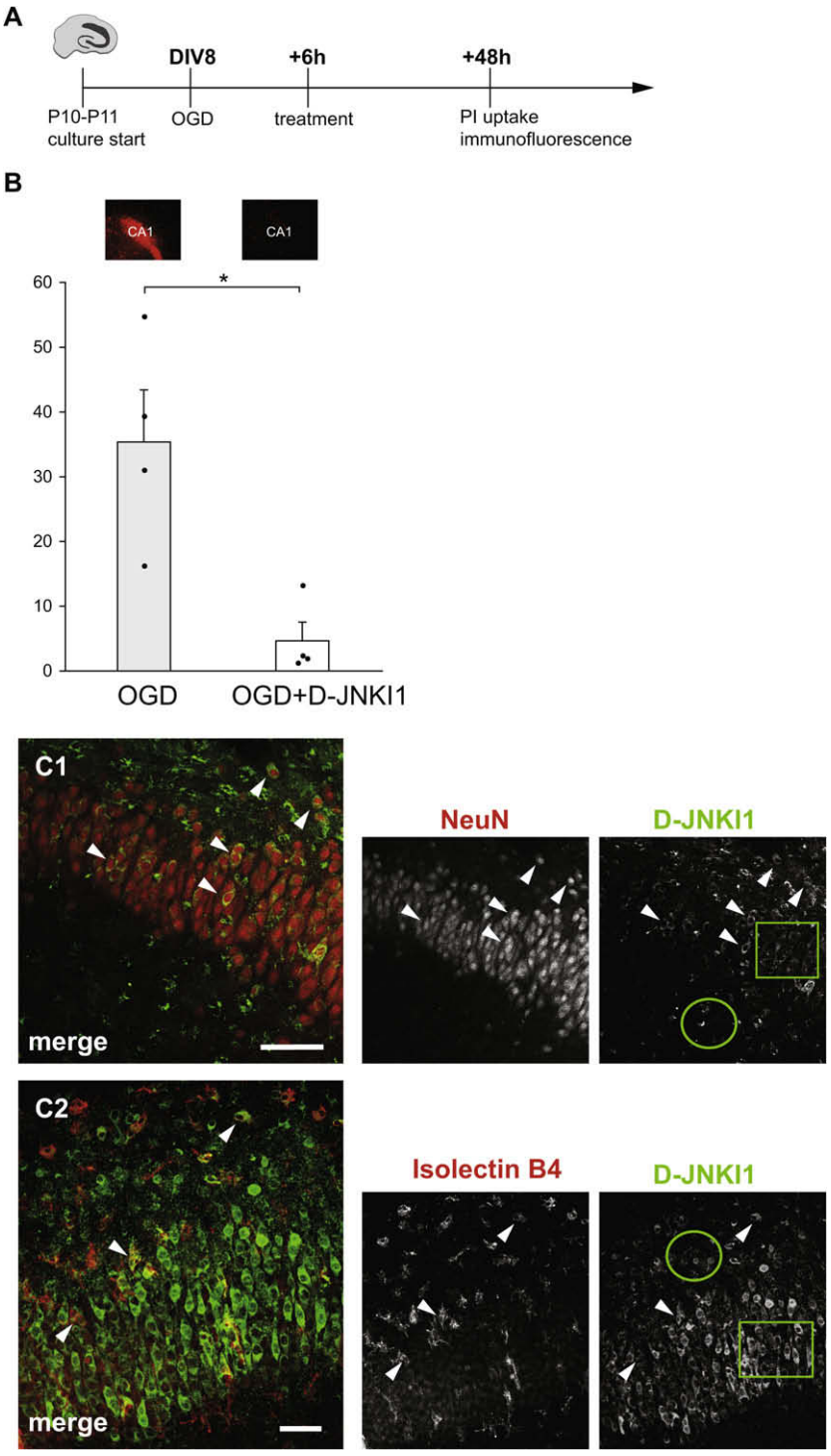

Fig. 5. Co-localization of D-JNKI1 in neurons and microglial cells 48 h after OGD in hippocampal slices. (A) Schematic representation of the experimental protocol. OGD for 30 min was performed 8 days after *in vitro* culture, 12 nmol/l D-JNKI1 or vehicle was administrated in the culture medium 6 h after OGD. PI uptake and immunofluorescence was analyzed 48 h after induction of ischemia. (B) Effect of D-JNKI1 on neuronal death assessed by cellular PI uptake. Representative photomicrographs showing PI uptake in the CA1 layer of hippocampal slice cultures not exposed (left) and exposed (right) to vehicle or D-JNKI1, respectively. A significant decrease of PI uptake was observed 48 h after D-JNKI1 treatment. Diagram shows percent of cell death in the CA1 region in both groups ($p = 0.012$; $^{*}p < 0.05$). Control slices not exposed to OGD had no PI uptake (data not shown). (C) D-JNKI1 is mainly localized in the CA1 pyramidal cell layer of hippocampal slices 48 h after OGD and shows a cytoplasmic staining (outlined by green squares), whereas few stainings are localized outside the CA1 region (green circles). Co-localization of D-JNKI1 (green cytoplasmic staining) with NeuN (red nuclear labelling) is found in treated slices, predominantly in the CA1 (C1, arrow heads). Some co-localization was also observed between D-JNKI1 and Isolectin B4-positive microglial cells at the border of the CA1 layer (C2, arrow heads).

As we observed activation of p-c-Jun in neurons and not in microglia, and that D-JNKI1 was mainly localized in neurons but also in some microglial cells, we may assume that D-JNKI1 is more likely to indirectly modulate microglial activation *in vitro*.

Intriguingly, the *in vivo* results suggest that treatment with D-JNKI1 following ischemia does not reduce the recruitment and activation of microglia in the ischemic brain territory at 48 h. However, D-JNKI1 appears to alter microglial activation and

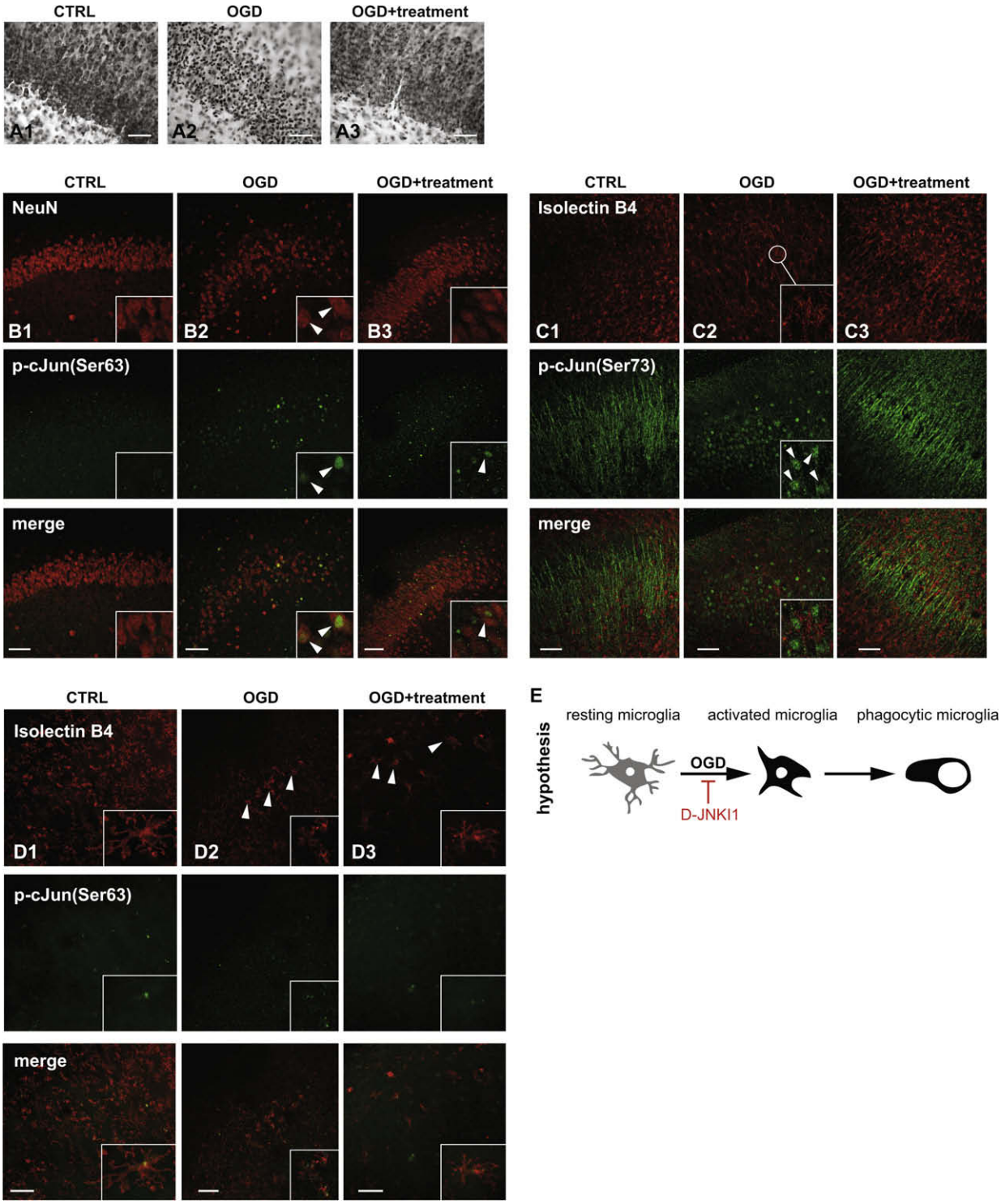

Fig. 6. Activation of c-Jun after OGD and effect of D-JNKI1 on microglia activation. (A) Nissl-staining of the CA1 region of control (CTRL), OGD and OGD + D-JNKI1 slices. CA1 neurons are undergoing ischemic cellular death, shown by pyknotic nuclei (A2), whereas treated slices (A3) as well as control slices (A1) show neurons in a healthy state; scale bar: 50 μm. (B) After OGD nuclear expression of p-c-Jun(Ser63) co-localized with NeuN-positive cells (arrows), while NeuN staining is partially lost (B2) compared to control slices (B1). D-JNKI1 (12 nmol/l) treatment rescues NeuN staining while it partially attenuates p-c-Jun staining (B3). In both OGD (C2) and OGD + D-JNKI1 (C3) groups, no Isolectin B4 cells co-localized with p-c-Jun(Ser73) (arrow heads). (D) In control slices microglial cells are highly ramified and are evenly distributed (D1, red). After OGD microglia accumulates in the damaged CA1 layer and changes its morphology to an activated state (ameboid microglia) (D2, arrow heads), whereas in treated slices, morphology of microglial cells are heterogeneous: ameboid (D3, arrow heads) and ramified cells (D3, insert), and are localized at the border or within the neuronal CA1 layer, respectively. Inserts show high magnification. (E) Schematic presentation of microglial morphological transformation in response to an ischemic insult such as OGD *in vitro*. Our initial hypothesis is that after an insult resting microglia become highly activated, further accumulate and could change morphology to a phagocytic state. We suggest that D-JNKI1 may inhibit the activation of microglia and secretion of pro-inflammatory mediators. We postulate that part of this inhibition process may be mediated through the JNK pathway and lead to neuroprotection. (B–D) All fluorescent microphotographs of double labelling are from the CA1 hippocampal region. Scale bar: 100 μm.

localization following OGD *in vitro* (Fig. 6E: schematic presentation). While this may partly be due to the *in vitro* hypoxia model not reproducing the *in vivo* ischemia model, these observations need to be further explored, for instance by quantitative measurements of inflammatory cytokines released by microglia, in animals submitted to experimental ischemia with or without treatment.

4. Discussion

In the normal healthy brain, microglial cells are known to continually screen their microenvironment by quickly moving their processes (Kreutzberg, 1996; Nimmerjahn et al., 2005). Controlled activation of microglia induces production of trophic factors and elimination of cell debris, leading to a protection of surrounding cells (Raivich et al., 1999; Streit et al., 1999; Aloisi, 2001). An ischemic insult causes an exaggerated activation of microglia with excessive release of pro-inflammatory mediators and uncontrolled phagocytic function (van Rossum and Hanisch, 2004; Kreutzberg, 1996). It has been postulated that this over-activation might be deleterious for the survival of neurons whereas controlled activation of microglia might be neuroprotective.

The purpose of this study was to investigate whether the strong neuroprotective effect of D-JNKI1 in our models of cerebral ischemia was due to a modulation of microglia activation through the JNK pathway. We found that D-JNKI1 was strongly neuroprotective when injected i.v. 3 h after transient 45 min MCAo. Despite efficiently attenuating the damaging neuronal events by inhibition of the JNK pathway (Waetzig et al., 2005; Raivich and Behrens, 2006), D-JNKI1 did not reduce the accumulation and morphological activation of microglia *in vivo* as shown by our quantification of CD11b+ cells (Fig. 4). This result does not completely rule out that D-JNKI1 could inhibit microglia at earlier steps, as CD11b labelling was positive at 24 h after MCAo. Alternately, as D-JNKI1 was not detected in the brain, it could also possibly inhibit peripheral JNK activation, which could lead to reduced inflammatory processes and indirect neuroprotection. These findings show that neuroprotection due to D-JNKI1 treatment in this model is independent of the peak of microglial activation observed at 48 h.

Furthermore, our analysis of linear regression between the infarct volume and the number of microglia in the ischemic tissue of vehicle-treated animals reveals that there is a strong correlation: the larger the lesion, the more activated microglial cells are present in the infarct. Treatment with D-JNKI1 does not significantly change this trend. Therefore, D-JNKI1 inhibits neuronal death without affecting the accumulation of microglia, nor by having an obvious effect on microglial activation state, suggesting that activation of microglia does not necessarily exacerbates neuronal damage. As microglia are capable of various responses depending on the type of injury, and since changes in morphology and upregulation of surface expression markers does not always reflect microglial activation, it will be necessary to go onto investigate the inflammatory capacity in this model (Feuerstein et al., 1998; Hanisch, 2002; Garden and Moller, 2006; Clausen et al., 2008). Previous studies have reported that ischemic damage is attenuated by inhibiting pro-inflammatory cytokines or transcription factor such as nuclear factor-κB (NF-κB), by diminishing free radicals generated by inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2) (Iadecola et al., 1995; Yang et al., 1998; Parmentier et al., 1999; Nijboer et al., 2009). As the JNK pathway is involved in regulation of inflammatory molecules (Hidding et al., 2002; Waetzig et al., 2005), D-JNKI1 might therefore modulate cytokines secretion. Investigating pro- and anti-inflammatory mediators with or without D-JNKI1 treatment will complete our understanding of microglial function after ischemia.

Several studies have described the involvement of the mitogen-activated protein kinase pathway (MAPKs, including JNK, ERK and p38) in the regulation of inflammatory mediators (for review see Kaminska, 2005). *In vitro* experiments have shown that the JNK pathway is activated in microglial cultures stimulated by TNFα, thrombin or LPS (Hidding et al., 2002; Waetzig et al., 2005). They also showed that CEP-11004 (an inhibitor of mixed lineage kinases) or SP600125 (an ATP-competitive JNK inhibitor) reduced LPS-induced phosphorylation of JNK and c-Jun and significantly reduced microglial metabolic activity and proliferation, respectively. Here, we report for the first time that phospho-c-Jun was activated in microglia after transient MCAo in mice, which led us to test whether D-JNKI1 could modulate microglial activity through the regulation of the JNK pathway. However, JNK may not be the only predominant pathway required for microglial activation in the brain after cerebral ischemia (Fig. 4). As all three MAPKs are upregulated after stress (Kaminska, 2005), one could imagine that inhibition of more than one pathway is necessary for complete inhibition of microglial activation. An inhibitor of p38 mitogen-activated protein kinase (SB239063) has been shown to reduce microglial activation after OGD in hippocampal cultures when administrated 2 h before and until 24 h after the deprivation (Strassburger et al., 2008). Thereby combined inhibitors of the MAPK pathways could affect more significantly the activation of microglia *in vivo*.

Towards an understanding of the molecular mechanisms of the JNK pathway in microglia, we investigated the role of D-JNKI1 *in vitro*. In our OGD model of ischemia, we showed that the peptide prevented neuronal cell death in the CA1 by a reduction of propidium iodide uptake, as previously described (Hirt et al., 2004). We found a strong activation of phospho-c-Jun in CA1 neurons 48 h after OGD. Treatment with D-JNKI1 did not completely block c-Jun activation but rescued neurons from OGD-induced death.

In slices subjected to OGD and treated with D-JNKI1, we find co-localization of D-JNKI1 with neurons and to a much lesser extent with microglia. Interestingly, we observed morphological changes of microglia *in vitro*: while microglial cells were in a well ramified state in control slices, they became more rounded after OGD, and D-JNKI1 inhibited this morphological change. The JNK pathway modulates the phosphorylation of actin and microtubules and could thereby modify microglial morphology (Waetzig et al., 2005). Our *in vitro* observations suggest an effect of D-JNKI1 on microglia and could be further confirmed by assessing microglial function, such as cytokine release. The JNK pathway was for instance shown to be involved in microglial pro-inflammatory cytokine production (Kaminska, 2005) and we have started to investigate cytokine secretion *in vitro* after OGD as well as *in vivo* after MCAo.

Besides modification of microglial morphology after D-JNKI1 treatment *in vitro*, we found a change in microglial localization after OGD and after OGD + D-JNKI1. In control slices microglia were homogenously distributed whereas after OGD microglia were predominantly localized within the damaged neuronal CA1 layer. Treatment with D-JNKI1 modified the localization of microglia which were found at the boundary of the neuronal CA1 layer. Modification of microglial cell localization at the site of injury has been previously reported in hippocampal slice cultures (Heppner et al., 1998; Montero et al., 2009). Altogether, OGD induced changes in microglial morphology (ramified to ameboid) and accumulation in the degenerating neuronal CA1 layer. These changes were modified by D-JNKI1. We may hypothesize that D-JNKI1 exerts a neuroprotective effect directly on neurons (shown in this *in vitro* study by localization of D-JNKI1 in NeuN-positive cells and by previous data in neuronal cultures, Borsello et al., 2003), and modifies the intracellular signaling from affected neurons to microglia. Thus,

D-JNKI1 would act indirectly on microglia through signals secreted by neurons influencing microglial activity and localization.

Interestingly, the hippocampal slice hypoxia model may not reproduce mechanisms of microglial activation observed in the *in vivo* stroke model. Indeed, D-JNKI1 showed no effect on microglial activation and accumulation at 48 h after MCAo, while changes in morphology and localization were observed *in vitro*. This may reflect species or age differences (adult mice used *in vivo* versus newborn rats *in vitro*) which might cause discrepancies. Furthermore, *in vitro* models are only partially representative of the *in vivo* situation concerning the microglial response. Indeed, we saw DCs and infiltration of inflammatory cells, such as T lymphocytes after MCAo (Fig. 1), which evidently does not occur *in vitro*. These cells could modulate microglial activation and may explain the differences seen in the two models. The different effects observed in the models deserve further investigation to highlight the possibility that neuronal injury and microglial activation may happen independently.

Here we demonstrated that D-JNKI1, a selective JNK inhibitor and highly neuroprotective peptide, does not attenuate the activation and accumulation of microglia in the ischemic tissue in an *in vivo* MCAo model of stroke at 48 h. This finding adds to the knowledge that neuroprotection via inhibition of JNK is independent of the late microglial accumulation or activation. It also indicates that the JNK pathway is not essential to microglial activation after ischemia, and that the presence of activated microglia in the ischemic area is not necessarily detrimental to neurons. Additionally, our study suggests that D-JNKI1 could be safely used in clinical stroke patients, as it does not exacerbate inflammatory responses after cerebral ischemia. Our *in vitro* data point towards an effect of D-JNKI1 on microglial recruitment as well as morphological changes, which is likely to be an indirect effect of the peptide through its action on neurons. Therefore, investigating the pattern of pro- and anti-inflammatory secreted mediators in brain ischemia with and without D-JNKI1 treatment will help to characterize its role on inflammation during cerebral ischemia.

Disclosure

Dr. C. Bonny is employed by Xigen Pharmaceuticals. Dr. L. Hirt has acted as consultant for Xigen Pharmaceuticals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2009.11.001.

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